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THE UNIVERSITY OF ALBERTA

Leaf Epicuticular Wax of Canola: Ultrastructure, Chemistry and Interaction with *Alternaria brassicae*

by

Kenneth Lyle Conn

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Plant Pathology

Department of Plant Science

EDMONTON, ALBERTA

Spring 1986

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ISBN 0-315-30135-X

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Leaf Epicuticular Wax of Canola: Ultrastructure, Chemistry and Interaction with Alternaria brassicae submitted by Kenneth Lyle Conn in partial fulfilment of the requirements for the degree of Master of Science in Plant Pathology.

.....
Supervisor

.....

.....

Date Dec. 31, 1985

Abstract

Alternaria brassicae is an important pathogen worldwide and causes the black spot disease of canola. Generally, Brassica napus is less susceptible to this disease than B. campestris, and the amount of epicuticular wax is at least one of the factors involved in this difference. This study explored the effects of wax on the retention and germination of the conidia of A. brassicae on leaves of the canola cultivars Candle, Tobin (B. campestris), Altex and Westar (B. napus). Wax ultrastructure and chemistry were investigated to determine if they were also involved in the different susceptibility of the two host species.

The leaf epicuticular wax in canola appeared to confer lower susceptibility to A. brassicae in at least three ways. By creating a hydrophobic surface, the wax reduced the retention of conidia, and perhaps by impeding the movement of exudates, it reduced the germination rate of the conidia and the number of germ tubes produced by each conidium. The wax was not found to have any fungistatic effect.

There were no appreciable differences in the ultrastructure or the chemical composition of the waxes among the four cultivars. Their surfaces were covered with an evenly distributed layer of wax crystals superimposed on an amorphous layer of wax. Some trends such as the density the wax on the leaves and fruits appeared to be species specific, whereas the density of the wax on the stems did not. The density of wax was high on the stems of all four

cultivars. Also, the younger leaves of B. campestris had a much higher density of wax than the older leaves. The amount of wax on the leaves was determined by extracting the wax. Brassica napus had on an average more than twice as much wax as B. campestris. There appeared to be at least three types of wax crystals present. These included plate-like crystals, filamentous, sometimes branched crystals, and rods, present singly or forming blocks. Their waxes consisted of nine major classes of constituents. These included alkanes, esters, ketones, aldehydes, sec-alcohols, ketols, prim-alcohols, triterpenols and fatty acids. The major constituents of the waxes were C_{29} alkane, C_{29} ketone, C_{29} sec-alcohol and C_{46}-C_{44} esters.

The freeze-drying and the air-drying methods of specimen preparation for SEM examination of the wax layer were compared. The freeze-drying method resulted in disruption and washing away of the wax crystals, whereas there was no visible damage with the air-drying method.

The lesser susceptibility of B. napus to A. brassicae relative to that of B. campestris, appears to be, at least in part, due to higher amounts of epicuticular wax and not to any other differences in ultrastructure or chemistry of the waxes.

Acknowledgments

I wish to express my sincere gratitude to my supervisor Dr. J. P. Tewari for his valuable guidance and encouragement during the course of my research program and for providing financial support. I would also like to thank my committee members for their assistance, in particular, Dr. D. Hadziyev for his help in the chemical analysis of wax and Dr. B. P. Dancik for the use of his gas-liquid chromatograph.

I especially want to thank my parents for their encouragement and understanding during the course of this work.

Table of Contents

Chapter — Page

I. General Introduction 1

A. Preface 1

1. Cultivation of Canola (Rapeseed) 1

2. Alternaria Black Spot Disease 2

B. Roles of Plant Epicuticular Wax 5

1. Plant Surface Wettability 5

2. Cuticle Permeability 6

3. Water Balance (Transpiration) 6

4. Defence Against Plant Disease 7

a) Antimicrobial Effect of Wax 7

b) Effect of Wax on Plant Leachates 9

5. Other Roles of the Wax 10

C. Ultrastructure and Formation of Epicuticular Wax 13

1. Ultrastructure of the Wax 13

2. Biogenesis of the Wax 14

3. Effect of Environmental Conditions on Wax Production 19

4. Methods Used to Study the Morphology of the Wax Layer 21

D. Chemistry of Plant Epicuticular Wax 23

1. Chemistry of the Wax 23

2. Methods Used to Extract the Wax 25

3. Relationship Between Chemistry and Ultrastructure 26

4. Use of Plant Epicuticular Wax for Chemotaxonomy 28

E. Objectives of the Thesis 29

II. Leaf Surface - Interactions with *Alternaria brassicae* 30

A. Introduction 30

B. Materials and Methods 31

1. Plant Material 31

2. Fungal Material 31

3. Preparation for Conidial Germination Experiments 32

4. Recrystallization of Wax 33

C. Results and Discussion 35

1. SEM of Wiped Leaf Surface 35

2. Major Sources of Variability Between Experiments 35

3. Effect of Leaf Epicuticular Wax on Conidial Retention 36

4. Effect of Leaf Epicuticular Wax on Conidial Germination 38

5. Effect of Leaf Epicuticular Wax on the Number of Germ Tubes 41

6. Effect of Recrystallized Wax on Conidial Germination 42

D. Conclusions 43

E. Figures and Legends 44

III. Ultrastructure of Canola Epicuticular Wax 69

A. Introduction 69

B. Materials and Methods 70

1. Plant Material 70

2. Preparation of Plant Material for SEM 70

a) Method A: Freeze-drying 70

b) Method B: Air-drying 71

C. Results and Discussion 72

1. Morphology of Canola Epicuticular Wax 72

2. Density of Leaf Epicuticular Wax 76

3. Density of Stem Epicuticular Wax 78

4. Density of Fruit Epicuticular Wax 78

D. Conclusions 80

E. Figures and Legends 81

IV. Chemistry of Canola Leaf Epicuticular Wax 106

A. Introduction 106

B. Materials and Methods 107

1. Plant Material 107

2. Extraction of Wax 107

3. Fractionation of Wax 107

a) Thin Layer Chromatography 107

b) Semi-Preparative Layer Chromatography 108

c) Column Chromatography 110

4. Infrared Spectroscopy 110

5. Gas-Liquid Chromatography 111

C. Results and Discussion 112

1. Amount of Epicuticular Wax on Canola Leaves 112

2. Chemistry of Canola Leaf Epicuticular Wax 113

a) Major Classes of Constituents 113

b) Major Constituents 114

D. Conclusions 118

E. Figures and Legends 119

V. General Discussion and Conclusions 132

A. Canola Epicuticular Wax versus A. brassicae ...132

B. Suggestions for Future Studies 138

Bibliography 140

Appendix: Infrared Spectra for the Nine Classes of Constituents from the Fractionated Leaf Epicuticular Wax of Altex. 150

List of Tables

Table — Page

1. Principal Classes of Constituents Occurring in Plant Epicuticular Waxes. 24

2. Germination of Conidia of A. brassicae on Recrystallized Wax of Canola. 42

3. Amounts of Wax on the Leaves of Canola. 112

4. Percent Composition of the Major Wax Classes from the Leaf Epicuticular Wax of Altex. 115

List of Figures

Figure — Page

1. Schematic Representation of the Structure of a Leaf Cuticular Membrane. 17

2. SEM of a Wiped Leaf Surface. 46

3. Comparison of Bloom Between Rapeseed Cultivars. 48

4. Hydrophobic Effect of Leaf Epicuticular Wax. 50

5. Effect of Leaf Epicuticular Wax on Retention of the Conidia of A. brassicae. 52

6. Effect of Leaf Epicuticular Wax on the Amount of Infection by A. brassicae. 54

7. Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae (expt. 1). 56

8. Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae (expt. 2). 58

9. Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae (expt. 3). 60

10. Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae. 62

11a. Effect of Leaf Epicuticular Wax on the Formation of Germ Tubes by the Conidia of A. brassicae. 64

11b. Effect of Leaf Epicuticular Wax on the Formation of Germ Tubes by the Conidia of A. brassicae. 66

11c. Effect of Leaf Epicuticular Wax on the Formation of Germ Tubes by the Conidia of A. brassicae. 68

12. A. Top view of the adaxial surface of a middle leaf of Candle, prepared for SEM by method B.
B. Oblique view of the adaxial surface of a middle leaf of Candle, prepared for SEM by method B.
C. Top view of the adaxial surface of an upper leaf of Candle, prepared for SEM by method A. 83

13. A. Top view of the adaxial surface of a middle leaf of Westar, prepared for SEM by method B.
B. Oblique view of the adaxial surface of a middle leaf of Westar, prepared for SEM by method B.
C. Top view of the adaxial surface of an upper leaf of Westar, prepared for SEM by method A. 85

14. A. Top view of the adaxial surface of a middle leaf of Tobin.
B. Oblique view of the adaxial surface of a middle leaf of Tobin.
C. Oblique view of the adaxial surface of an upper leaf of Tobin. 87

15. A. Top view of the adaxial surface of a middle leaf of Altex.
B. Oblique view of the adaxial surface of a middle leaf of Altex.
C. Oblique view of the adaxial surface of an upper leaf of Altex. 89

16. A. Top view of the abaxial surface of an upper leaf of Altex.
B. Oblique view of the abaxial surface of an upper leaf of Altex.
C. Oblique view of the fruit surface of Westar from the upper part of the inflorescence. 91

17. A. Top view of the stem surface of Westar from the middle of the plant.
B. Oblique view of the stem surface of Westar from the middle of the plant.
C. Oblique view of the fruit surface of Westar from the lower part of the inflorescence. 93

18. A. Top view of the stem surface of Candle from the middle of the plant.
B. Oblique view of the stem surface of Candle from the middle of the plant.
C. Oblique view of the fruit surface of Candle from the middle of the inflorescence. 95

19. A. Top view of the stem surface of Altex from the middle of the plant.
B. Oblique view of the stem surface of Altex from the middle of the plant.
C. Oblique view of the fruit surface of Altex from the upper part of the inflorescence. 97

20. A. Top view of the stem surface of Tobin from the middle of the plant.
B. Oblique view of the stem surface of Tobin from the middle of the plant.
C. Oblique view of the fruit surface of Tobin from the upper part of the inflorescence. 99

21. A. Adaxial surface of an upper leaf of Westar prepared for SEM by method A, showing a sheet of wax that has been turned upside down.
B. Adaxial surface of an upper leaf of Westar prepared for SEM by method A, showing dislodged wax

crystals.
C. Adaxial surface of an upper leaf of Altex showing plate-like wax crystals.
D. Adaxial surface of an upper leaf of Tobin showing filamentous wax crystals.
E. Adaxial surface of a middle leaf of Tobin showing a branched filamentous wax crystal. 101

22. A. Stem surface of Altex from the middle of the plant showing rods.
B. Abaxial surface of an upper leaf of Tobin showing fused rods.
C. Adaxial surface of an upper leaf of Westar showing fused rods. 103

23. A. Stem surface of Westar from the middle of the plant showing plate-like crystals and fused rods.
B. Stem surface of Altex from the middle of the plant showing the wax crystals at different stages of development. 105

24. Separation of Leaf Epicuticular Wax into Major Classes of Constituents by TLC. 121

25. GLC-trace for Candle Leaf Epicuticular Wax. 122

26. GLC-trace for Tobin Leaf Epicuticular Wax. 123

27. GLC-trace for Altex Leaf Epicuticular Wax. 124

28. GLC-trace for Westar Leaf Epicuticular Wax. 125

29. GLC-trace for the Alkane Fraction from Altex Leaf Epicuticular Wax. 126

30. GLC-trace for the Ester Fraction from Altex Leaf Epicuticular Wax. 127

31. GLC-trace for the Ketone Fraction from Altex Leaf Epicuticular Wax. 128

32. GLC-trace for the Sec-Alcohol Fraction from Altex Leaf Epicuticular Wax. 129

33. GLC-trace for the Aldehyde Fraction from Altex Leaf Epicuticular Wax. 130

34. GLC-trace for the Prim-Alcohol Fraction from Altex Leaf Epicuticular Wax. 131

Chapter I

General Introduction

A. Preface

1. Cultivation of Canola (Rapeseed)

Rapeseed is grown worldwide largely for the edible oil and meal content of the seed and is a major cash crop in western Canada. In western Canada two major species of rapeseed are grown. One of them is Brassica campestris L., also referred to as summer turnip rape or Polish rapeseed, and the other one is Brassica napus L., also referred to as summer rape or Argentine rapeseed. Over the years, Canadian plant breeders have improved the quality of rapeseed by decreasing the erucic acid and glucosinolate contents of the seed (Martens et al., 1984). The name canola has been given to specific cultivars of rapeseed which have oil that is low in erucic acid (<5%) and meal that is low in glucosinolate (<3 mg/g of moisture-free and oil-free meal) (Vaisey-Genser and Eskin, 1982). Most of the rapeseed cultivars now grown in Canada are of canola quality. Throughout this thesis both the terms rapeseed and canola will be used.

The fruits of canola are commonly called pods, but the correct term is siliques. The term pod refers to a fruit which has one carpel and one locule, usually with one dehiscing suture, like that of the legume family. A silique refers to a fruit which has two carpels and two locules,

with the pericarp dehiscing into two halves, leaving the membranous septum with seeds on it. This type of fruit is found in the rapeseed family. Many cultivars and landraces of rapeseed grown in the Orient depart from this general type in having one to several locules. However, all cultivars of rapeseed grown in Canada have fruits that are bilocular. Throughout this thesis the general term fruits will be used instead of the terms siliques or pods.

2. Alternaria Black Spot Disease

The fungi Alternaria brassicae (Berk.) Sacc. and A. raphani Groves & Skolko are found worldwide (Weiss, 1983) and cause a disease on rapeseed known as Alternaria Black or Grey Spot. These fungi are capable of causing the disease together or alone. Alternaria brassicae is the species that has been isolated most frequently from diseased material in Alberta. A. brassicicola (Schw.) Wilts. causes a similar disease which is important in Europe and the United States (Petrie, 1975). It is found in Canada only sporadically on garden crucifers. The requirement of higher temperatures for fast spore germination appears to impede the prevalence of this pathogen in western Canada (Degenhardt et al., 1982). A strain of another species, A. alternata (Fr.) Keissler, from Alberta is also reported to be capable of causing leaf spotting and floral blight in B. campestris (Vaartnou and Tewari, 1972). This thesis involved the study of A. brassicae only.

Alternaria Black Spot disease is present every year, wherever rapeseed is grown, and under proper moisture conditions can cause severe damage. This is a major disease of rapeseed in Canada (Degenhardt et al., 1974; Petrie, 1973). Degenhardt et al. (1974) reported experimental yield losses of 34 to 70% depending on the host-pathogen combinations.

The symptoms of the disease include lesions on leaves, stems and fruits. Lesions start as small grayish-black specks which enlarge to become spots with grayish-black, often concentrically zonate centers, surrounded by chlorotic areas. These lesions cause accelerated senescence of the leaves and reduce the photosynthetic area of the plant, which in turn reduces the vigor of the plant. Heavy infections can result in severe defoliation. Fruits with infected pedicels fail to develop and drop off. Severely infected fruits contain shrunken and infected seeds. The infected fruits dry, shrink and shatter easily when harvested, dropping the seed to the ground. Infection reduces the yield of oil extracted from the seed and reduces the protein content of the meal (Degenhardt et al., 1974). If infected seed is planted, pre- or post-emergence damping-off can occur. The hypocotyls and cotyledons of surviving seedlings may be infected with conidia produced on damped-off seedlings (Martens et al., 1984; Nyvall, 1979).

Alternaria brassicae produces large multicelled conidia (spores) and under unfavorable conditions can also form

microsclerotia and chlamydospores (Tsuneda and Skoropad, 1977). Although conclusive evidence is still lacking, these structures may allow the fungus to overwinter on infected canola debris and on seed. Infections are caused by the pathogen sporulating on the seed and on plant debris or in lesions on living plants. Also, several cruciferous weeds are hosts on which the fungus can potentially overwinter. Since the conidia of A. brassicae are large, they are probably not dispersed for long distances by wind. Their spread from plant to plant is most likely by splashing rain droplets.

This disease can be controlled by sowing disease-free seed, rotation of rapeseed for a minimum of three years with non-cruciferous crops, plowing under infected residue, and by controlling volunteer rapeseed and cruciferous weeds. There is no fungicide presently registered in Canada specifically for use against A. brassicae. However the antibiotics Polyoxin B and D have been found to be very effective in controlling A. brassicae on rapeseed (Tewari and Skoropad, 1979). Nectria inventa Plowr. may possibly be used as a biological control agent for A. brassicae. Nectria inventa is a destructive mycoparasite of A. brassicae that suppresses its vegetative growth and sporulation (Tsuneda and Skoropad, 1978).

B. Roles of Plant Epicuticular Wax

1. Plant Surface Wettability

When a droplet of water sits on a leaf surface, the contact angle between the leaf surface and the water droplet is determined by the surface tension forces between the two. The contact angle is smaller between a droplet of water and a hydrophilic surface, and larger between a droplet of water and a hydrophobic surface. The larger the contact angle, the lower the wettability of a surface.

It has been found that contact angle measurements correlate well with the degree of glaucousness of a plant surface, and indicate the types of wax structure present (Hall et al., 1965; Holloway, 1969b; Merrall, 1981; Netting and von Wettstein-Knowles, 1973; Tewari and Skoropad, 1976; Troughton and Hall, 1966). Glaucous leaves that have contact angles greater than 120° have many deposits of rodlet or platelet type wax which stands out from the leaf surface (Hall et al., 1965). Contact angles of less than 110° indicate that the droplet is in contact with a more hydrophilic cuticle surface (Hall et al., 1965). If the wax components are considered individually, the alkanes are the most hydrophobic, with esters, ketones and secondary alcohols being closely behind (Holloway, 1969a).

2. Cuticle Permeability

It has been demonstrated that the decreased wettability of a glaucous plant surface decreases the permeability of the cuticle. Norris and Bukovac (1974) demonstrated that removal of wax from pear leaves increased the penetration of naphthaleneacetic acid into the cuticle. Norris (1974) demonstrated that removal of the wax from leaves of tomato increased the penetration of 2,4-D. Thus, the epicuticular wax impedes the uptake of foliar-applied chemicals (Hunt and Baker, 1982). To increase the absorption, spreading, cutting and sticking ability of herbicides, chemicals known as surfactants are frequently added (Cantliffe and Wilcox, 1972; Kuzych and Meggitt, 1983). Kuzych and Meggitt (1983) demonstrated that surfactants accomplish this by altering the epicuticular wax structure.

One method used to demonstrate the effect of wax on permeability has been the use of chemicals that inhibit wax formation. Flore and Bukovac (1981) used a pesticide (S-ethyl dipropylthiocarbamate) to inhibit epicuticular wax production on the developing leaves of cabbage, resulting in an increase in cuticular permeability.

3. Water Balance (Transpiration)

The decreased permeability of the cuticle due to epicuticular wax reduces transpiration. Schönherr (1976) demonstrated that the removal of wax from isolated cuticular membranes of pear leaves increased their water permeability

by a factor of 300 to 500. Grncarevic and Radler (1967) demonstrated that the epicuticular wax on grapes decreased transpiration, and that the alcohol, hydrocarbon and aldehyde fractions of the wax were the components involved.

Wax is very important in regulating plant transpiration (Denna, 1970a,b; Hamilton and Hamilton, 1972). Some plants, such as carnations, are grown from shoot-tip cultures, and it has been found that under these conditions epicuticular wax production is decreased (Sutter and Langhans, 1982). It has been suggested that desiccation after transfer of plants from *in vitro* conditions may be the result of poor epicuticular wax formation (Fuchigami *et al.*, 1981; Sutter and Langhans, 1982).

4. Defence Against Plant Disease

The role of plant epicuticular wax in providing disease resistance is important in many diseases. The physical and chemical characteristics of the leaf surface are the first barriers encountered by an invading foliar pathogen (Hargreaves *et al.*, 1982; Martin and Juniper, 1970).

a) Antimicrobial Effect of Wax

Blakeman and Atkinson (1981) reviewed the information on antimicrobial substances associated with the aerial surfaces of plants. They pointed out that in the majority of reports, the site of origin of isolated compounds had not been determined. In many cases, it was not known whether active compounds had been isolated

from the epicuticular wax, the cuticle as a whole, or from internal tissues. They pointed out that there was great variability in the extraction techniques used and that this made the comparison of data from different sources very difficult. However, there have been some studies which demonstrated that constituents of certain epicuticular waxes were antimicrobial. Fatty and resin acids from needle epicuticular wax of Pinus radiata D. Don were shown to be highly fungistatic against Dothistroma pini Hulbary (Franich and Gadgil, 1983). The compounds inhibited both spore germination and mycelial growth in vitro. The epicuticular wax from the berries and leaves of Coffea arabica L. was shown to be toxic to Colletotrichum coffeanum Noack, the causal organism of coffee berry disease (Lampard and Carter, 1973). Two antifungal isoflavones, luteone and wighteone, were found to be associated with the leaf epicuticular wax of Lupinus albus L. and may have a role in preventing the penetration of the cuticle by A. brassicicola and Botrytis cinerea Pers. ex. Fr. (Harborne et al., 1976; Hargreaves et al., 1982). Martin et al. (1957) found apple leaf wax to be fungistatic against the powdery mildew Podosphaera leucotricha and reduced the germination of the conidia of this pathogen. The surface wax from the leaves of beetroot was found to inhibit the germination of B. cinerea spores (Blakeman and Atkinson, 1976; Blakeman

and Szejnberg, 1973). Sharma (1984) studied the chemistry of the leaf epicuticular wax in several cultivars of rapeseed grown in India that show differences in susceptibility to A. brassicae and A. brassicicola. Sharma (1984) found differences in the chemistry of the waxes among cultivars and indicated the possibility that this was involved in the resistance of some cultivars to these fungi. However, there was no evidence provided to link the differences in resistance to the differences in chemistry of the wax.

b) Effect of Wax on Plant Leachates

The reduction in wettability and permeability of the cuticle by the epicuticular wax also reduces the amount of leaching from a plant (Blakeman, 1973; Tukey, [illegible]0, 1971). It has been demonstrated that both organic [illegible]rganic substances accumulate in water that is in [illegible] plant surfaces. Some of these materials [illegible] outside the plant as deposits from the [illegible] mineral particles, pollen grains and rain [illegible] 1976). A greater proportion of these [illegible], originate from within the plant and pass [illegible] cuticle into water in contact with the surface [illegible] 1976). This movement of plant exudates is referred to as leaching. The leachates include a large number of substances such as simple sugars, all amino acids known to occur in plants, organic acids, growth regulators, vitamins, alkaloids,

phenols and all the essential minerals (Blakeman, 1973).

It is known that leachates can be utilized by both saprophytic and parasitic micro-organisms (Blakeman, 1973). For example, anthranilic acid in the leachates from banana fruits was found to stimulate germination and appressorium formation by Colletotrichum musae(Berk. and Curt) Arx (Harper and Swinburne, 1979). Germination of the conidia of B. cinerea was stimulated by sucrose and fructose present in the leachates from grape berries (Kosuge and Hewitt, 1964).

Thus, by reducing the movement of plant exudates, the epicuticular wax may indirectly reduce the susceptibility of the plant to disease.

A method of disease control that is being considered, is the spray application of waxes and plastic polymers to enhance the effect of the epicuticular wax. Ziv and Frederiksen (1983) evaluated several waxes and plastic polymers as protectants against foliar pathogens on maize, sorghum and wheat. They found that these products reduced the severity of several diseases (anthracnose, leaf blight, rust, downy mildew and powdery mildew).

5. Other Roles of the Wax

Krause and Houston (1983) found that epicuticular wax was involved in SO_2-tolerance of white pines. They found

that SO_2-tolerant clones had epicuticular wax covering the stomata of the needles, whereas in SO_2-sensitive clones there was a split or crack in the wax covering the stomata. The cracks may enhance gaseous exchange, which would result in increased SO_2 absorption (Krause and Houston, 1983).

Haines et al. (1985) found that leaf wettability may be an important determinant of foliar damage by acid rain. They found a positive correlation between leaf wettability and susceptibility to acid rain.

The epicuticular wax may also provide protection against UV radiation. It is known that the epidermal cells are damaged by enhanced UV radiation (Hall et al., 1975; Tevini et al., 1981). It was shown that the structural arrangement of the wax increased the reflectance of the UV-B radiation as well as visible light (Clark and Lister, 1975; Robberecht and Caldwell, 1980). Steinmüller and Tevini (1985) demonstrated that enhanced UV-B levels caused an increase in the total wax in barley, bean and cucumber leaves by ~25%. This may indicate that plants respond to enhanced UV by producing more epicuticular wax for protection.

It has been suggested that glaucousness may protect a plant from frost damage. Barber (1955) studied Eucalyptus sp. and found a correlation between the degree of glaucousness in an area and the frost activity there, with the more glaucous plants occurring in frost prone localities. He suggested that this may be a result of

natural selection. However, there was no direct evidence provided to link glaucousness to frost hardiness.

The epicuticular waxes of many plants affect the feeding behavior of insects (Woodhead, 1983). Many insects select plants for feeding on the basis of the chemistry of the leaf surface. Woodhead (1983) demonstrated that the epicuticular wax of young Sorghum bicolor leaves contained a number of chemicals that deterred the feeding by Locusta migratoria L. Thus the epicuticular wax of some plants protects them from insects.

C. Ultrastructure and Formation of Epicuticular Wax

1. Ultrastructure of the Wax

Plant epicuticular wax is composed of an amorphous layer upon which may be superimposed crystalline structures in the form of plates, tubes, ribbons, rods, filaments or dendrites (Baker, 1982). These wax crystals form a "fluffy" layer on top of the amorphous layer of wax. Baker and Parsons (1971) were able to demonstrate a thin layer of wax on the cuticular surface of Brassica oleracea L. This was studied by cross-hatching the leaf surface with a wire and then observing the leaf by Scanning Electron Microscopy (SEM). Many of the crystals that comprise the "fluffy" layer project away from the leaf surface forming a "forest" of wax crystals. If this layer of wax is dense enough, it causes the leaves to be glaucous imparting a bluish coloration or "bloom" to the leaf surface due to the reflecting properties of the wax. Plants may possess heavy deposits of wax, but if the wax is amorphous or has only little crystalline structure, they are not termed as glaucous, but are referred to as being waxy.

The ultrastructure of plant epicuticular wax has been reviewed by a number of authors (Baker, 1982; Caldicott and Eglinton, 1973; Hadley, 1981; Hamilton and Hamilton, 1972; Martin and Juniper, 1970). In many plants the "fluffy" layer of wax consists mainly of a single type of wax crystal (Baker, 1982). For example, there are predominantly plates

on Pisum sativum L. leaves (Baker and Holloway, 1971), tubes on Ginkgo biloba L. leaves (Jeffree et al., 1976), ribbons on Fragaria ovalis leaves (Baker and Parsons, 1971) and filaments on Eucalyptus globulus Labill. leaves (Baker and Parsons, 1971). However in Brassica the "fluffy" layer of wax is usually a composite of wax crystals. For example, the leaf surface of B. oleracea contains rods and filaments (Hall et al., 1965). The types of wax crystals that have been reported on rapeseed include plates, rods and tubes (Armstrong and Whitecross, 1976; Holloway et al., 1977; Tewari and Skoropad, 1976; Whitecross and Armstrong, 1972; Wortmann, 1965).

2. Biogenesis of the Wax

An intriguing and unresolved question with respect to the epicuticular wax is how the wax passes through the plant cuticle to reach the surface. It is known that wax is synthesized in the epidermal layer of cells, and reaches the surface as soon as it is synthesized (Kolattukudy, 1970). Mueller et al. (1954) observed that the distribution of wax deposits on B. oleracea leaves was approximately constant during leaf expansion, suggesting that new wax deposits are interposed between the older deposits as the epidermal cells multiply and expand. For the wax to reach the surface, it has to pass through the cellulose wall of the epidermal cells, through a pectin layer, through a mixed layer of cutin, wax and carbohydrate polymers, and finally, through a

cutin and wax layer (Fig. 1). As the wax migrates to the surface some of it impregnates the cutin layers of the cuticle.

Four theories have been put forth to explain the movement of wax. The first theory proposes that the plant cuticle contains pores or channels through which the wax components or wax precursors pass (Baker, 1982). A few studies, using SEM and Transmission Electron Microscopy (TEM), have provided evidence of pores and channels in some plants, but many more have failed to find any evidence of them (Baker, 1982). It is possible that in the few plant cuticles in which pores and channels can be seen, wax does pass through them, but since pores or channels are not universally found in plants, this theory cannot be generalized.

A second theory is the liquid extrusion theory which holds that wax is extruded under pressure through the cuticle in the form of a soft paste (Baker, 1982). However this theory is not very likely for two reasons. The first is that for wax to be extruded as a soft paste, there would have to be channels in the cuticle, which are not present in most plants. The second reason is that wax components have relatively high melting points, which makes it unlikely that wax could be retained in a semi-solid state prior to extrusion (Baker, 1982).

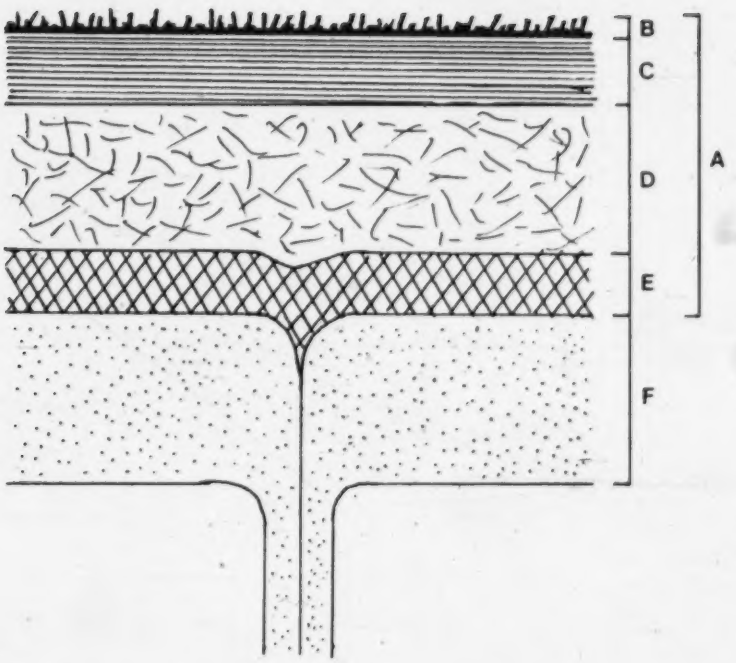
A third theory is the polymerization theory in which wax precursors formed in the cell diffuse through the

Figure 1

Schematic Representation of the Structure of a Leaf Cuticle (A).

B Epicuticular wax
C Cutin and wax
D Cutin, wax and carbohydrate polymers
E Pectin
F Cellulose wall of epidermal cells

Adapted from Hadley (1981), Jeffree *et al.* (1976) and Kolattukudy (1970).


B
C
D
E
F
A

cuticle and polymerize at the surface to form the wax crystals (Baker, 1982). However, barring a few exceptions, polymeric constituents have not been identified in plant waxes (Baker, 1982). Also this theory proposes that newly exuded wax pushes the polymeric material away from the plant surface leaving the most recent wax at the base of the crystalline structure, but it has been shown by Baker (1974) that wax crystals can form at the tops of existing rods and tubes.

A fourth theory is the crystallization theory in which wax components are carried through the cuticle in a volatile solvent and crystallize at the plant surface (Baker, 1982). Research on the recrystallization of extracted wax is providing direct evidence for this theory. It has been shown that under appropriate conditions wax is capable of organizing itself into crystalline structures independently of the underlying cells. Jeffree et al. (1975) used a model system which showed that extracted wax can crystallize into wax crystals with shapes and dimensions similar to those on the intact plant surface. They also showed that when the wax was fractionated into its different components, and the fractions recrystallized sequentially, the resulting crystals more closely resembled those on the intact leaf surface, than when the whole wax was recrystalized. This indicated that the wax constituents may be secreted to the surface in a definite sequence, or perhaps be delivered at different sites, rather than as a homogenous mixture. From

recrystallization experiments it was found that the morphology of wax crystals was also influenced by the rate of crystallization (Jeffree et al., 1976).

Thus, it may be that after wax synthesis, the epidermal cells and cuticle play no further role in the development of the ultrastructure of plant surface waxes. The fact that extracted wax can be recrystallized in a form identical to that on the plant surface is evidence against any chemical modification of the wax at the plant surface after excretion. The crystallization theory assumes that the epidermal cells contain enough volatile solvents to carry the wax to the surface, but it is not known if this is the case. The carrier solvents would have to be organic solvents because the wax is insoluble in an aqueous solution. Some possible solvents that have been suggested are short-chain aldehydes, ketones, alcohols, and 1-hexane (Tulloch, 1976).

3. Effect of Environmental Conditions on Wax Production

The type, size and distribution of epicuticular wax crystals can be significantly modified by environmental conditions. Environmental factors such as temperature, light intensity, humidity and soil moisture can affect the quantity of wax and its ultrastructure. Armstrong and Whitecross (1976) and Whitecross and Armstrong (1972) showed that leaves of B. napus were more densely covered with wax at higher temperatures and light intensities. Wardle et al.

(1983) demonstrated that plantlets of B. oleracea cultured under reduced humidity developed larger quantities of surface wax. Hunt and Baker (1982) demonstrated that pea leaves had increased wax production in response to decreased soil moisture content and increases in vapour pressure deficit. Darnell and Ferree (1983) showed that the production of epicuticular wax on apple leaves increased as soil water potential decreased. These increases in wax production indicate the importance of wax in reducing water loss in plants. The ultrastructure of wax can also be modified by environmental factors. Armstrong and Whitecross (1976) and Whitecross and Armstrong (1972) found that as the temperature was increased there was a transition in the ultrastructure of the wax on leaves of B. napus, from rods at day/night temperatures of 15°C/10°C to wax platelets at higher temperatures of 27°C/22°C. An increase in the proportion of platelets to rods would decrease the water loss from the leaves. Baker (1974) found that increases in light intensity increased the size of the wax tubes in B. oleracea.

It appears that the environmentally induced changes in wax structure occur more readily in epicuticular waxes that are structurally complex than in those in which a single component is dominant (Hunt et al., 1976; Jeffree et al., 1976). This would mean that the epicuticular wax in Brassica species is more sensitive to environmental factors than that in other plants with structurally less complex

waxes.

4. Methods Used to Study the Morphology of the Wax Layer

Most of the earlier studies on the ultrastructure of epicuticular wax were done using TEM of carbon replicas of the wax surface. While in some cases these techniques revealed the types of wax crystals present (Davis, 1971), these techniques cannot demonstrate the orientation of wax crystals on the surface. Then, SEM techniques were developed which revealed better the ultrastructure of the wax. There have been many SEM techniques used. Parsons et al. (1974) and Falk et al. (1971) compared many different techniques for SEM and found that they varied greatly in their results. This has led at times to conflicting interpretations of the leaf surface. Falk et al. (1971) also found that osmium tetroxide vapor was the best chemical fixative for this purpose.

A more recent technique used is cryostage SEM, in which the SEM is fitted with a cold stage and the specimen is kept at a very low temperature. However, Jeffree and Sandford (1982) demonstrated that care should be taken when using cryostage SEM because the specimen can become contaminated with granular coatings of ice if special precautions are not taken. They pointed out that this has led to wrong interpretations of wax ultrastructure by some researchers. Also, in conventional instruments, the cryostage could act as a cold trap for the other SEM column contaminants, such

as the vacuum system oil vapour, or vapourized carbon, leading to further artifacts. To minimize this effect an ultra-high and clean vacuum would be required.

D. Chemistry of Plant Epicuticular Wax

1. Chemistry of the Wax

The chemistry of plant epicuticular wax has been reviewed by a number of authors (Baker, 1982; Caldicott and Eglinton, 1973; Hadley, 1981; Hamilton and Hamilton, 1972; Kolattukudy, 1970, 1975, 1980; Tulloch, 1976). Wax is composed of complex mixtures of mainly long-chain aliphatic compounds comprising a number of classes. Table 1 shows the major classes of constituents occurring in plant epicuticular waxes. These classes can be further grouped according to whether their major homologues are comprised primarily of even numbers of carbon atoms, odd numbers of carbon atoms, or are cyclic homologues (Table 1). Many minor wax constituents have also been identified. Epicuticular wax chemistry has been studied for many different plants and appears to be distinctive for each plant type. Only a few Brassica species have been studied so far. These include cabbage (Flore and Bukovac, 1978; Holloway and Brown, 1977; Macey and Barber, 1970; Netting et al., 1972; Schmid and Bandi, 1971), cauliflower (Holloway and Brown, 1977), broccoli (Holloway and Brown, 1977), brussel sprouts (Baker and Holloway, 1975) and rapeseed (Holloway and Brown, 1977; Holloway et al., 1977). Holloway et al. (1977) studied three lines of B. napus (Nilla, a Nilla mutant and a Rigo mutant) and separated the leaf epicuticular waxes into nine major classes of compounds.

Table 1. Principal classes of constituents occurring in plant epicuticular waxes.

Component class	Homologue range	Most common constituent
Dominant even carbon-number		
Acids (short series)	C_{12}-C_{24}	hexadecanoic(C_{16}); octadecanoic(C_{18})
Acids (long series)	C_{20}-C_{32}	hexacosanoic(C_{26}); octacosanoic(C_{28})
Aldehydes	C_{22}-C_{32}	hexacosanal(C_{26}); octacosanal(C_{28}); triacontanal(C_{30})
Primary alcohols	C_{22}-C_{32}	hexacosanol(C_{26}); octacosanol(C_{28}); triacontanol(C_{30})
Alkyl esters	C_{36}-C_{52}	hexacosyl(C_{26}) and octacosyl(C_{28}) esters of hexadecanoic(C_{16}), octadecanoic(C_{18}), eicosanoic(C_{20}) and docosanoic acids(C_{22})
Dominant odd carbon-number		
Hydrocarbons	C_{21}-C_{33}	nonacosane(C_{29}); hentriacontane(C_{31}); tritriacontane(C_{33})
Secondary alcohols	C_{29}-C_{33}	nonacosanol(C_{29}); hentriacontanol(C_{31})
Ketones	C_{29}-C_{33}	nonacosanone(C_{29}); hentriacontanone(C_{31})
β-diketones	C_{29}-C_{33}	hentriacontane-14,16-dione(C_{31}); tritriacontane-16,18-dione(C_{33})
Hydroxy β-diketones	C_{29}-C_{33}	derivatives of hentriacontane-14,16-dione(C_{31})
Cyclic constituents		
Triterpenoid acids		ursolic acid; oleanolic acid
Triterpenols		β-amyrin; α-amyrin; lupeol
Triterpenoid esters		β-amyrenyl acetate; taraxeryl acetate; lupeyl acetate
Triterpenoid ketones		taraxerone; lupene-3-one

Adapted from Baker (1982)

These included alkanes, esters, ketones, aldehydes, secondary alcohols, ketols, primary alcohols, triterpenols and fatty acids. The relative proportions of each class were different for each of the three lines.

2. Methods Used to Extract the Wax

Chloroform, hexane or petroleum ether have been the main solvents used for the extraction of epicuticular wax. The extraction techniques however, have varied considerably from one study to another. A distinction that has to be made when extracting wax is whether only the epicuticular wax is to be extracted, or the intracuticular wax as well, because their compositions are not necessarily the same (Baker, 1982). Haas and Rentschler (1984) studied the composition of the epicuticular and intracuticular waxes of blackberry leaves and found them to be very different. If the extraction technique is too vigorous, all of the plant waxes would be extracted. In some investigations the researchers were interested in all the waxes in a plant and stated so. They used extraction techniques such as placing leaves of a myrtaceous species in warm petroleum ether for five minutes (Courtney et al., 1983) and Sargassum fulvellum fronds in methylene chloride for 90 days (Miyazawa et al., 1982). In other investigations the researchers indicated that they were only interested in the epicuticular wax, but used extraction techniques that may have been too vigorous. Some examples of these cases include placing Chionochloa

shoots in petroleum ether for 16 hours (Cowlishaw et al., 1983), watercress in ethyl ether for 30 minutes (Spence and Tucknott, 1983), Epacridaceae leaves in petroleum ether for one to five minutes and flowers in petroleum ether for two or seven days (Salasoo, 1983) and orange tree leaves in chloroform at 55-58°C for 50 seconds (Freeman et al., 1979b). Some examples of more moderate extraction techniques for epicuticular wax are dipping mosses in chloroform at room temperature for 10 seconds (Hass, 1982), and grasses in hexane at room temperature for 10 seconds (Tulloch, 1983,1984). The extraction techniques needed would of course depend on the type of plants being studied and the types of epicuticular waxes present. More vigorous extraction techniques may be necessary for certain epicuticular waxes. However, it appears that some researchers may have used extraction techniques that were too vigorous, or should not have referred to the extracted wax as being the epicuticular wax fraction. The extraction techniques used would likely influence the results of a detailed chemical analysis of the wax. The variability in extraction techniques used would make comparison of data from different sources difficult.

3. Relationship Between Chemistry and Ultrastructure

Induced and spontaneous wax mutants lacking a normal glaucous surface have been used to study the relationship between wax ultrastructure and chemistry. These mutants can

be broadly classified into three types (Jeffree et al., 1976).

The first type includes mutants that have greatly reduced wax production caused by metabolic blocks in the synthesis of major wax components. This changes the chemical composition of the wax, and the plant surfaces may become non-glaucous due to the loss of the "fluffy" layer of wax crystals.

The second type of mutation causes changes in the chemical composition of the wax and the appearance of new types of wax crystals. These mutants provide good evidence that the chemistry of wax affects the ultrastructure. These mutants have a sub-glaucous appearance (Jeffree et al., 1976).

The third type of mutants includes the ones in which the quantity and chemical composition of the wax is unchanged but the ultrastructure is dramatically altered perhaps because of a change in the rate of wax formation. These mutants also have a sub-glaucous appearance (Jeffree et al., 1976).

From chemical and ultrastuctural studies of wax mutants it is apparent that the ultrastructure of wax is principally determined by the composition of the wax (Baker, 1982). Certain wax constituents have been associated with certain wax structures. For example, β-diketones have been associated with tubes (Freeman et al., 1979a; Wettstein-Knowles, 1974) and prim-alcohols with plates

(Wettstein-Knowles, 1974).

The study of mutants has shown that the process of epicuticular wax formation is ultimately under genetic control. In general, the ultrastructure and chemistry of the epicuticular wax are relatively stable characteristics of a plant species, and a species will exhibit identical wax ultrastructure and relatively constant chemical composition when grown under similar environmental conditions (Jeffree et al., 1976).

4. Use of Plant Epicuticular Wax for Chemotaxonomy

Plants are classified into many groups based on morphological characteristics. It has often been difficult, though, to distinguish between plant species which are morphologically very close. It may be possible to distinguish between these plants by comparing the chemistry of their epicuticular waxes. Some studies have been carried out that indicate the possibilities of developing a system of wax chemotaxonomy for certain plants (Baum and Tulloch, 1982; Cowlishaw et al., 1983; Mladenova et al., 1983; Salasoo, 1983; Tulloch, 1981,1983). It has been shown that the chemistry of the epicuticular wax is often distinctive for each species, subspecies, variety, etc. In future, the chemistry of plant epicuticular wax may prove to be a very useful tool in classification of morphologically similar plants.

E. Objectives of the Thesis

The objectives of this thesis were to determine the ultrastructure and chemistry of the epicuticular waxes in some of the current commercially grown Canadian cultivars of Brassica napus and B. campestris, and to investigate further the role of the epicuticular waxes in conferring resistance to Alternaria brassicae.

Chapter II

Leaf Surface - Interactions with *Alternaria brassicae*

A. Introduction

Most, if not all, members of the family Brassicaceae are susceptible to *A. brassicae* to varying degrees. The different cultivars of rapeseed show differences in susceptibility, but none of them are immune to this pathogen. Generally, the cultivars of *B. napus* are less susceptible than those of *B. campestris* and the amount of wax on the leaf surfaces has been shown to be at least one of the factors involved in this difference (Tewari and Skoropad, 1976; Skoropad and Tewari, 1977). The role of leaf epicuticular wax in rapeseed in providing a water-repellent surface has been demonstrated (Tewari and Skoropad, 1976). The epicuticular wax may contribute to lower susceptibility to *A. brassicae* in other ways as well. This study explores the effects of epicuticular wax on retention and germination of water-borne conidia of *A. brassicae* on the leaves of four currently grown commercial cultivars of rapeseed.

B. Materials and Methods

1. Plant Material

The cultivars of canola used were Tobin, Candle (B. campestris), Altex and Westar (B. napus). The plants were grown in soil in six inch pots with three plants per pot, in either growth cabinets at day/night temperatures of ~18°C/12°C and 16 hours light, or in the greenhouse where the conditions varied throughout the season. The plants in growth stage four (Harper and Berkenkamp, 1975) were used. The leaf immediately above the cotyledons was designated as the first leaf. Leaves from positions three to six were used for all experiments discussed in this chapter. This range of leaves was selected because the higher leaves were too small and the lower leaves were senescent.

2. Fungal Material

Alternaria brassicae was isolated from black spot lesions on the leaves of canola and was maintained by repeated subculturing on V8 juice medium containing rose bengal (Degenhardt et al., 1974). For experimental use it was grown on the same medium at 25°C in dark. Conidia were washed off the plates with distilled water, filtered through cheesecloth to remove clumps of hyphae, centrifuged, washed twice and resuspended in distilled water. Cultures used in experiments depicted in Figures 7 and 10 were five day-old, in Figures 9 and 11a,b,c, seven day-old, and in Table 2 and

Figures 5 and 8, 10 day-old. Suspensions of ~2 x 10^5 conidia/ml were used in experiments depicted in Figures 7 and 10 and ~3 x 10^5 conidia/ml in experiments depicted in Table 2 and Figures 5, 8, 9 and 11a,b,c.

3. Preparation for Conidial Germination Experiments

Leaves were excised from plants and placed in glass petri plates containing moist filter paper. The wax was gently wiped off one side of the midrib of each leaf with a moist cotton swab. In order to hold them flat, some of the leaves were stapled around their edges to the filter paper. The leaves were sprayed with a conidial suspension using a chromatographic sprayer held about one foot above the leaf. This delivered a fine mist of conidial suspension onto the leaves. At the same time, glass microscope slides in glass petri plates containing moist filter paper were sprayed to determine conidial germination in distilled water. Approximately 2 ml of the conidial suspension was sprayed per petri plate in experiments depicted in Figures 5 and 8, and ~1 ml for those depicted in Figures 7, 9, 10 and 11a,b,c. The petri plates were kept in continuous room light until at least 50% of the conidia on the glass slides germinated. This took two hours for the experiment shown in Figure 7, three hours for those in Figures 9 and 11a,b,c, and 6.5 hours for that in Figure 8. At that time, ethanol (2-3 ml) was added to each petri plate and the petri plates placed at 4°C until the percent germination could be

determined. This effectively stopped any further growth of the pathogen. To determine the percent germination, a piece of leaf (2 cm x 3 cm) was cut out from half way between the center of the midrib and the edge of the leaf for each half of leaf. The leaf pieces and glass slides were stained with lactophenol cotton blue and observed under the microscope (100x). At least 100 germinated and ungerminated conidia on each leaf piece or slide were counted. The germination experiment was repeated four times. In two experiments the number of germ tubes was also determined for the 100 conidia scored for germination. Also, for one experiment the total number of conidia retained was determined. This was done by placing three round cover slips (12 mm diameter) on the leaf pieces and slides. The total number of conidia were counted under the three cover slips (area of 3.37 cm^2).

To demonstrate the effect of wax on lesion development, the wax was removed from leaves as previously described. The leaves were then placed in glass petri plates with moist filter paper and sprayed with a conidial suspension, and kept in continuous room light for 48 hours.

A wiped leaf of Westar was examined with SEM after air-drying and coating with gold, to determine if wiping the wax off the leaves damaged the epidermal layer of cells.

4. Recrystallization of Wax

Wax extracted from the leaves of the four cultivars of rapeseed (see chapter IV for methodology) was recrystallized

on the frosted end of glass microscope slides. This was done by placing a few drops of wax dissolved in chloroform (20 mg/ml) on the frosted end of the slides, and immediatly placing the slides in an 80°C oven for 15 seconds to flash evaporate the chloroform. The slides were then allowed to air-dry for an hour. This resulted in a continuous layer of wax on the slides. If the chloroform was allowed to evaporate slowly, the wax recrystallized mainly around the edges of the slides. For the control, chloroform was placed on slides and evaporated as described above. The slides were then placed in glass petri plates containing moist filter paper and sprayed with a conidial suspension. The petri plates were kept in continuous room light until the germination on the control slides was at least 50%. At that time the slides were stained with lactophenol cotton blue and stored at 4°C until the percent germination could be determined. This experiment was repeated once.

C. Results and Discussion

1. SEM of Wiped Leaf Surface

Figure 2 shows a Westar leaf that had been wiped with a moist cotton swab. There appeared to be no mechanical damage to the epidermal cells. This indicated that the changes in percent germination and other effects that occurred upon wiping the wax from the leaves was not due to cell damage. With the naked eye, wiping appeared to remove most of the wax, but this micrograph shows that some wax was left behind. Most of the "fluffy" layer was removed but some wax plates remained. Also some smearing of wax could be seen on the surface (Fig. 2).

2. Major Sources of Variability Between Experiments

There appeared to be two major sources of variability between experiments. One was that the amount of bloom on the plants varied from one experiment to another. As discussed later, this variability would be expected to greatly affect the factors being studied. The other major source of variability was in the germination rates of A. brassicae conidia from one culture to another. The rate of growth of the cultures, the extent of sporulation, and the rate of germination of the conidia were variable. This made the time of observation of the percent germination different for each experiment. Due to these two major sources of variability, the results of the different experiments could

not be treated as replicates and the data pooled together, but rather had to be treated as separate experiments. However, neither of these two factors affected the general trends observed. The percentages presented in the figures should not be taken as absolute values, but rather as demonstrating trends. Thus, the data presented here represent results over a range of conditions and strengthen the validity of the trends observed.

Even though the amount of bloom varied from one batch of plants to another, the relative visible differences in the bloom between B. napus and B. campestris cultivars were constant. Brassica napus cultivars always had more bloom than B. campestris cultivars (Fig. 3).

3. Effect of Leaf Epicuticular Wax on Conidial Retention

The epicuticular wax creates a hydrophobic surface that decreases the retention of droplets of water. Figure 4 shows this effect for the four cultivars of canola studied. The right side of each leaf was wiped and the leaves sprayed with a fine mist of water. It can be seen that there was not much difference in the retention of water droplets between the wiped and unwiped surfaces of Candle and Tobin, but a considerable difference between these two surfaces was observed for Altex and Westar.

The hydrophobicity imparted by the wax layer should also affect the deposition of water-borne inoculum of A. brassicae. In order to study this aspect, the leaves were

sprayed with a conidial suspension, and the numbers of conidia deposited on the different leaf surfaces were determined.

Removal of the wax significantly increased the number of conidia that were deposited on the leaf surfaces for all four cultivars (Fig. 5). The retention of conidia on the unwiped leaf surfaces of Candle and Tobin was higher than that on Altex and Westar, because the leaf surfaces of Candle and Tobin have less wax. The retention of conidia on the wiped leaf surfaces of Candle and Tobin was also higher than that on Altex and Westar. Since Altex and Westar leaves had more wax to begin with, more wax may have remained behind after wiping. The retention of conidia on the glass slides was higher than that on the unwiped leaf surfaces of Altex and Westar, but lower than those in the rest of the treatments (Fig. 5).

This demonstrates the first important effect of wax, i.e., reduction in the retention of conidia. This will reduce the susceptibility of the more waxy plants to A. brassicae through a disease escape mechanism.

Figure 6 shows the differences that the removal of wax made on the amount of infection by A. brassicae. The right side of each leaf was wiped and the leaves sprayed with a conidial suspension. Visual assessment indicated that there was not much difference in the number of lesions between the wiped and unwiped surfaces of Candle and Tobin, but the two surfaces of Altex and Westar were very different.

4. Effect of Leaf Epicuticular Wax on Conidial Germination

Wiping the wax off the leaves significantly increased the rate of germination of the conidia for all four cultivars in most of the experiments. Figure 7 shows one experiment conducted with Candle, Altex and Westar, in which removal of the wax significantly increased the conidial germination on all three cultivars. The difference was greatest for Altex and Westar, but even the smaller difference in Candle was significant. Figure 8 shows another experiment conducted with Candle, Tobin, Altex and Westar, in which similar trends were observed. Removal of wax significantly increased the conidial germination on all four cultivars. This increase in germination rate may be due to the greater availability of leaf exudates, which may be stimulating the conidia to germinate faster. Thus, the wax indirectly may be affecting germination by reducing the diffusion of leaf exudates in droplets of water containing the conidia. This was also indicated by the fact that the germination rate of conidia on Candle and Tobin (wiped or unwiped leaves) and Altex and Westar (wiped leaves) was significantly higher than that in distilled water (Fig. 7). Also, the data shown in Figure 8 indicate that the wiped leaves of Candle, Tobin and Westar had a significantly higher germination rate of the conidia than that in distilled water. The smaller amounts of wax on Candle and Tobin may reduce the diffusion of exudates only to a small extent, whereas larger amounts of wax on Altex and Westar

may affect this to a much greater degree.

Figure 9 shows another experiment in which a similar trend in the rate of germination of the conidia could be seen, but the differences between wiped and unwiped leaves were not significantly different for Candle, Tobin and Westar, even though the differences between the means were actually larger than those in Figure 8. This could be due to the fact that the amount of wax on the leaves used was low, and that there was a large variability in the bloom within each cultivar. It was generally noted that the more waxy leaves had a lower percent germination than the less waxy leaves. This experiment demonstrated the effect of varying degrees of glaucousness on the rate of germination of the conidia.

Usually the percent germination on the wiped leaves of Altex and Westar was less than that on Candle and Tobin (Figs. 8 and 9). This can be explained by the fact that wiping did not remove all the wax and that more wax was left behind on Altex and Westar than on Candle and Tobin. Another explanation could be that there were more (or different) exudates diffusing from the leaves of Candle and Tobin than from those of Altex and Westar.

Another effect that can be seen in Figures 7, 8 and 9 was that the germination rate of the conidia on the unwiped leaves of Altex and Westar usually was significantly less than that in distilled water. This indicated that the wax might have some fungistatic effect. This result will be

discussed later in this chapter.

Figures 7, 8 and 9 show that the leaves with more wax supported slower germination of the conidia. The germination on all the wiped or unwiped leaves eventually reached 100%. However, this was delayed for several hours on the unwiped leaves of Altex and Westar. Figure 10 shows the germination of conidia on Candle, Altex, Westar and glass slides over a six hour period after inoculation. The data presented for two hours is the same as shown in Figure 7. It can be seen from this figure that the germination reached 100% on the wiped and unwiped leaves of Candle several hours before those of Altex and Westar. The germination rate on unwiped leaves of Altex and Westar was much slower than that on the wiped leaves.

In some experiments the conidial germination was followed by observations on the rates of penetration of the conidia and browning of host cells. It was observed that any differences in the germination rates were also reflected in the rates of penetration and browning of the host cells.

In addition to reducing the retention of water droplets containing conidia by providing a water repellent surface, the delay in the germination of the conidia and subsequent infection is another important effect of the wax. If a drop does stay on the leaf, the wax indirectly slows down the germination of the conidia, probably by impeding the diffusion of foliar exudates, thus decreasing the chances of infection. Also, on a waxy surface the drop or film of

water may dry up before the conidia can germinate and penetrate the leaf.

5. Effect of Leaf Epicuticular Wax on the Number of Germ Tubes

Removal of the wax significantly increased the number of germ tubes for all four cultivars of canola (Figs. 11a,b). The number of germ tubes on the wiped surfaces of Candle and Tobin was significantly higher than that on the glass slides (Fig. 11c). The number of germ tubes on the unwiped surfaces of Candle and Westar, and the wiped and unwiped surfaces of Altex, was significantly less than that on glass slides (Fig. 11c). Figure 11c also shows the means for the nine treatments, and the P values for the Chi-square analysis. Almost all the trends observed here were the same as those for the germination of the conidia. The increase in number of germ tubes was also probably due to the greater availability of leaf exudates upon removal of the wax.

The increase in number of germ tubes is the third important effect of the wax. When the number of germ tubes is increased, it should have the same effect as having more conidia present. The more germ tubes a conidium produces, the better are the chances of infection.

Thus, there appear to be at least three effects of the epicuticular wax, i.e., reducing conidial retention, reducing the germination rate of the conidia and reducing the number of germ tubes produced. If these factors are

considered individually, the reduction in conidial retention probably has the greatest impact on reducing susceptibity to A. brassicae. However, when these three factors are operational collectively, the effect of wax on A. brassicae may be greatly increased.

6. Effect of Recrystallized Wax on Conidial Germination

The recrystallized wax did not appear to affect the germination rate of the conidia. There was no significant difference in percent germination between the control slides and the slides with wax (Table 2).

Table 2

Germination of Conidia of A. brassicae on Recrystallized Wax of Canola.

Treatment	Germination(%)*
Control	53.7a
Candle	49.5a
Tobin	55.2a
Altex	53.7a
Westar	51.2a

* Percent germination determined 6.5 hours after inoculation.
Means are of six repeats.
Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

D. Conclusions

The leaf epicuticular wax of canola appears to confer lower susceptibility to A. brassicae in at least three ways. The first is that the wax creates a hydrophobic surface that decreases the retention of water-borne inoculum of A. brassicae. The second is that the germination of conidia is slower when a larger amount of wax is present, and the third is that the germ tubes produced by a conidium are fewer in number when a larger amount of wax is present. The last two effects are most likely due to the fact that larger amounts of wax may decrease the diffusion of leaf exudates into droplets of water containing conidia. These three factors acting together may significantly affect the extent of infection by A. brassicae in rapeseed. Also, with fewer germ tubes produced and slower germination, fewer conidia may have time to penetrate the leaf before the film or drop of water they are in dries up. There does not, however, appear to be any fungistatic effect of the wax on germination of the conidia.

E. Figures and Legends

Figure 2

SEM of a Wiped Leaf Surface.

A Westar leaf was wiped with a moist cotton swab, air-dried and examined with SEM.
Note that not all of the wax was removed. There were still some wax plates visible and some smearing of wax can be seen.
Such preparations indicated that the wiping did not damage the epidermal layer of cells (magnification x2200).

Figure 3

Comparison of Bloom Between Rapeseed Cultivars.

Shown are the plants of the cultivars Tobin, Candle (B. campestris), Altex and Westar (B. napus). Note that the B. napus cultivars are very bloomy and that the B. campestris cultivars are not.

Upper left - Altex
Upper right - Candle
Lower left - Westar
Lower right - Tobin

Figure 4

Hydrophobic Effect of Leaf Epicuticular Wax.

The right side of each leaf was wiped with a moist cotton swab and then the whole leaf was sprayed with a fine mist of water.
Note the low retention of water droplets on the unwiped surfaces of Altex and Westar.

Upper left - Altex
Upper right - Candle
Lower left - Westar
Lower right - Tobin

Figure 5

Effect of Leaf Epicuticular Wax on Retention of the Conidia of A. brassicae.

Data expressed as a percentage, with the highest treatment mean being considered as 100%.
Means are of six repeats.
The data were analysed before conversion to a percentage.
a-f. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

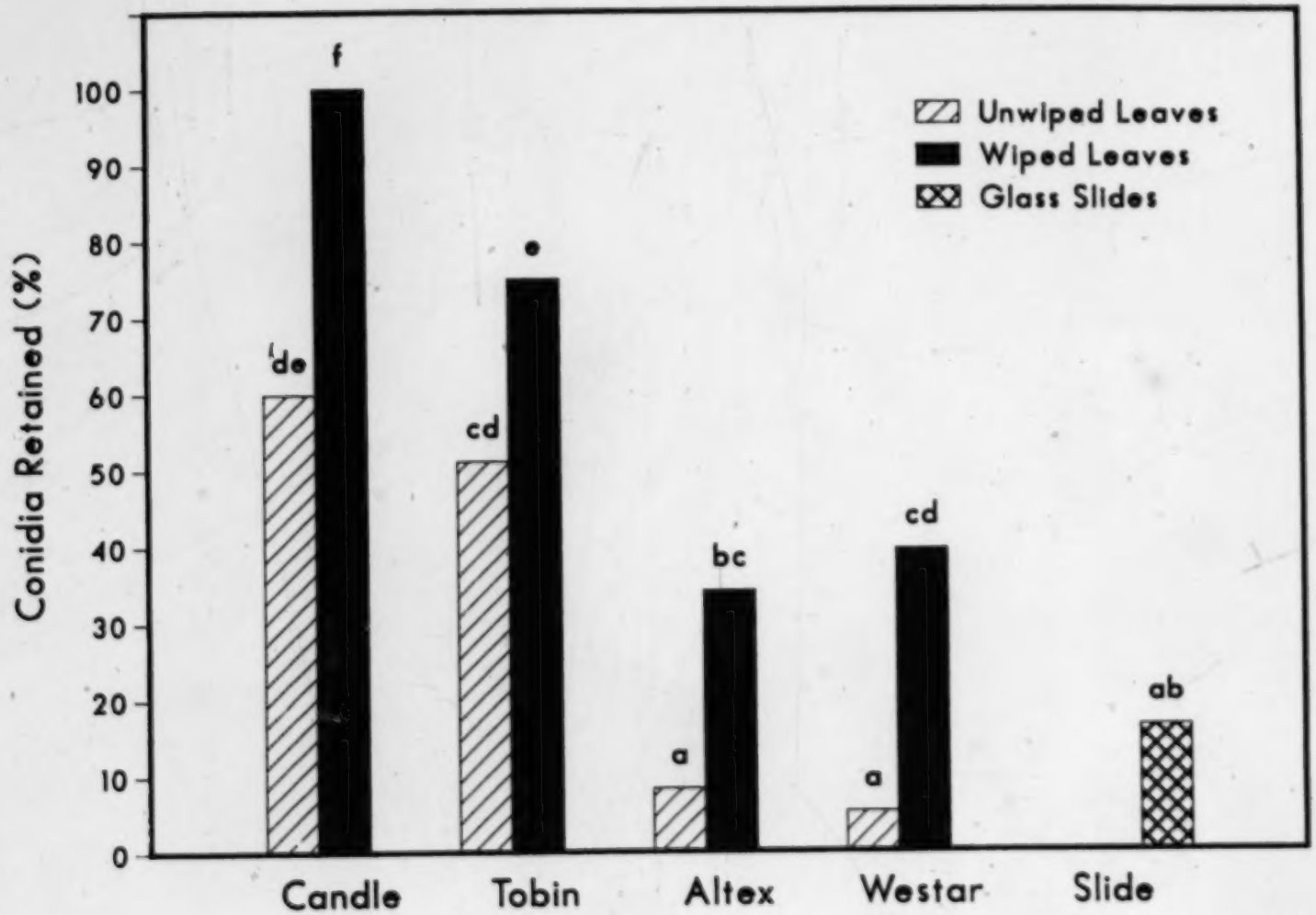

Conidia Retained (%)
100
90
80
70
60
50
40
30
20
10
0
f
de
cd
e
a
bc
a
cd
ab
Unwiped Leaves
Wiped Leaves
Glass Slides
Candle
Tobin
Altex
Westar
Slide

Figure 6

Effect of Leaf Epicuticular Wax on the Amount of Infection by A. brassicae.

The right side of each leaf was wiped with a moist cotton swab. The leaves were then sprayed with a conidial suspension, and kept under high humidity in continuous room light for 48 hours.
Note the sparse lesion development on the unwiped surfaces of Altex and Westar. All the leaves were green at the beginning of the experiment. The leaf chlorosis is associated with lesion development.

Upper left - Altex
Upper right - Candle
Lower left - Westar
Lower right - Tobin

Figure 7

Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae (expt. 1).

Percent germination was determined two hours after inoculation.
Means are of five repeats.
a-d. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

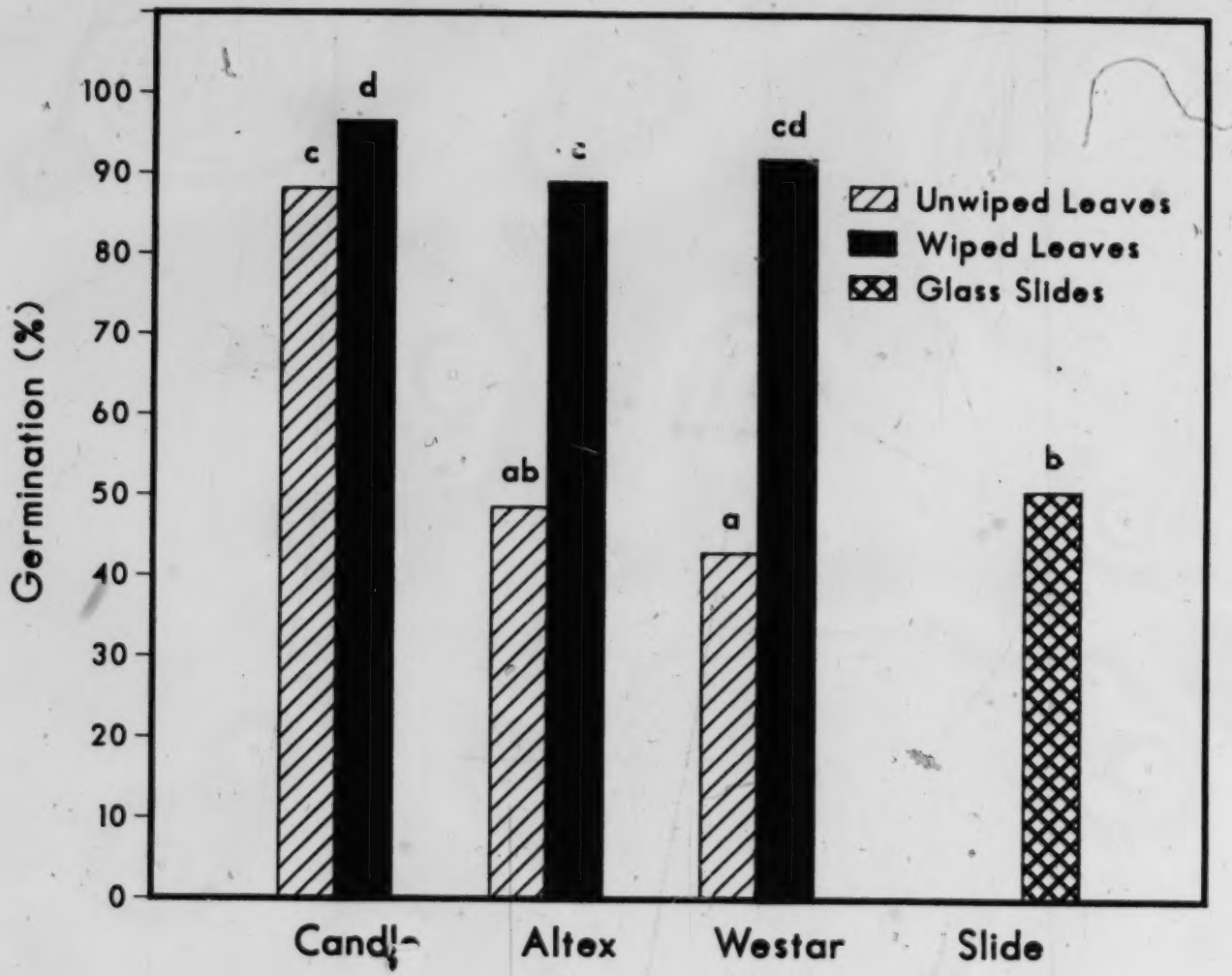

Germination (%)
100
90
80
70
60
50
40
30
20
10
0
c
d
ab
c
a
cd
b
Unwiped Leaves
Wiped Leaves
Glass Slides
Cand!-
Altex
Westar
Slide

Figure 8

Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae (expt. 2).

Percent germination was determined 6.5 hours after inoculation.
Means are of six repeats.
a-d. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

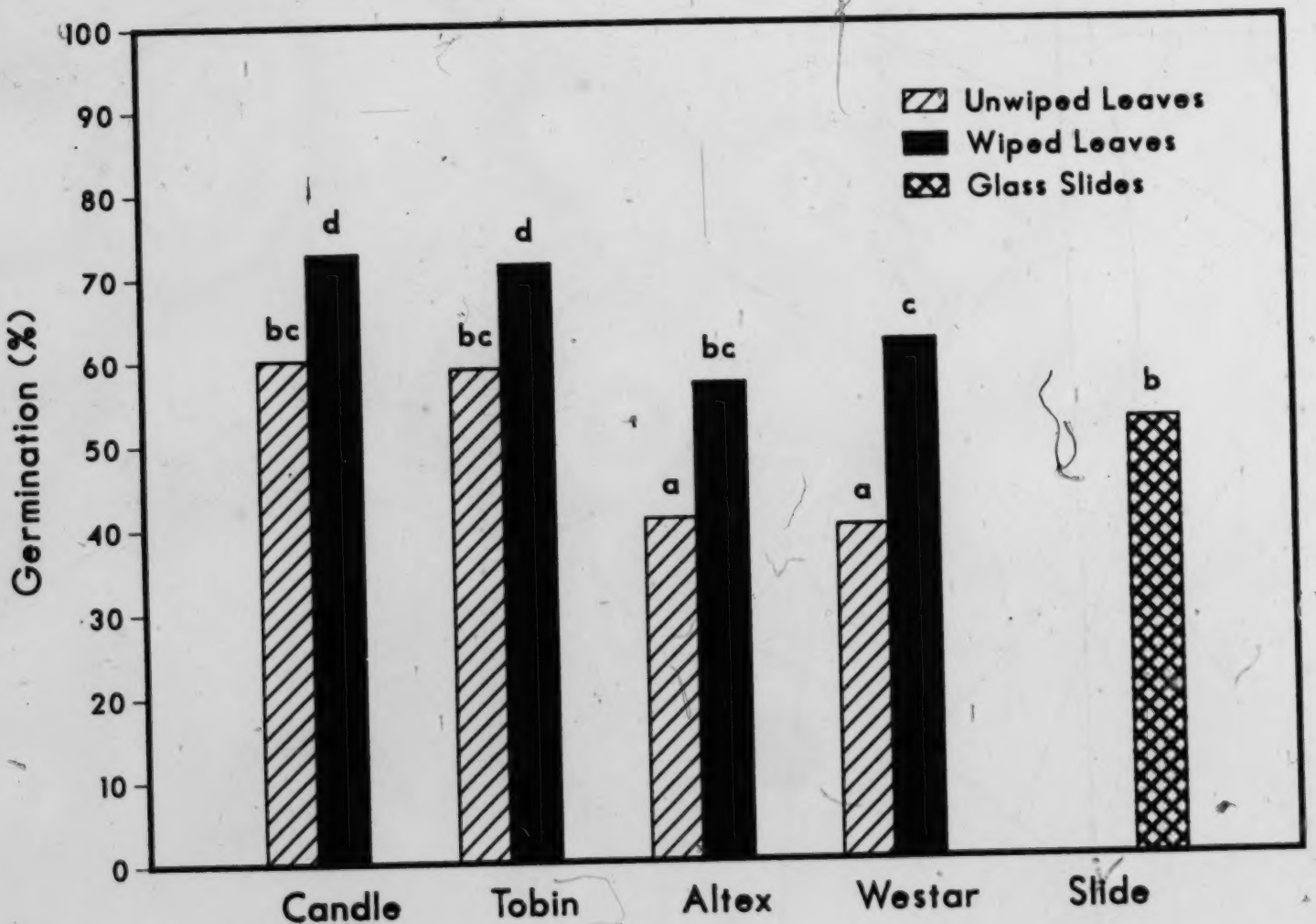

Germination (%)
100
90
80
70
60
50
40
30
20
10
0
Unwiped Leaves
Wiped Leaves
Glass Slides
bc
d
bc
d
a
bc
a
c
b
Candle
Tobin
Altex
Westar
Slide

Figure 9

Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae (expt. 3).

Percent germination was determined three hours after inoculation.
Means are of seven repeats.
a-c. Means sharing the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

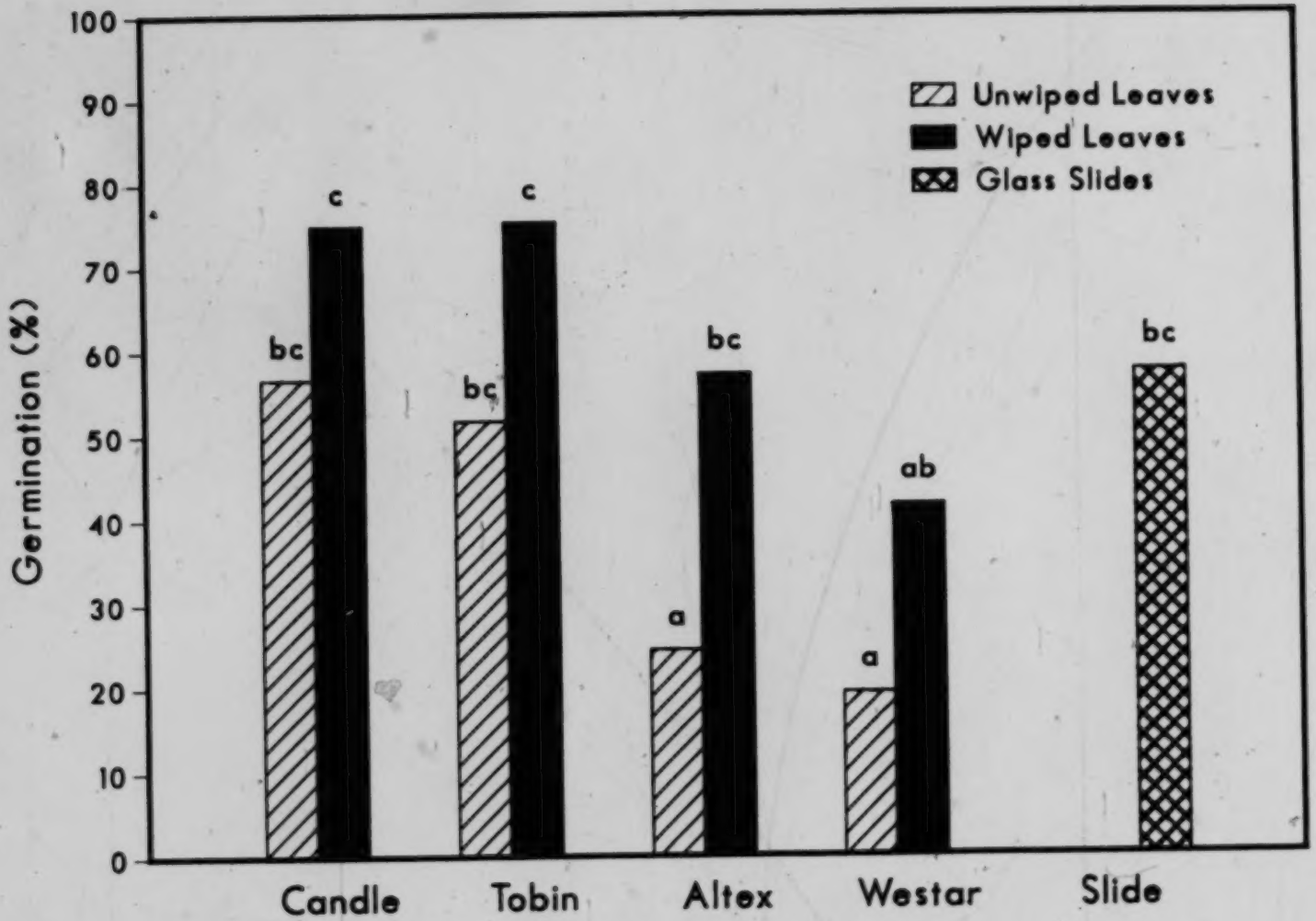
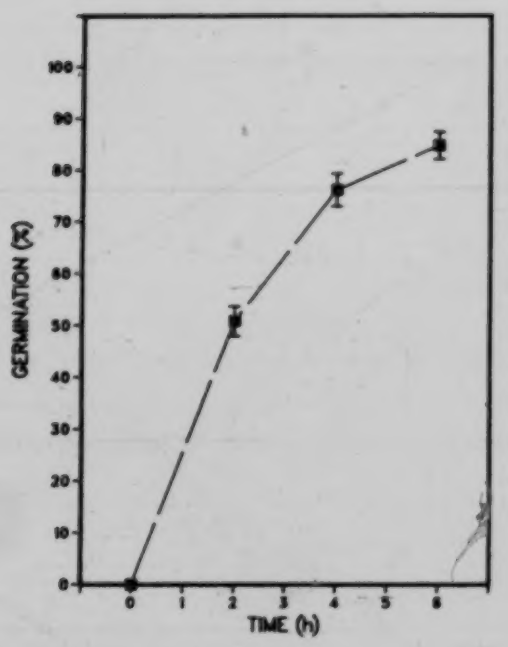

Germination (%)
100
90
80
70
60
50
40
30
20
10
0
Unwiped Leaves
Wiped Leaves
Glass Slides
bc
c
bc
c
a
bc
a
ab
bc
Candle
Tobin
Altex
Westar
Slide

Figure 10

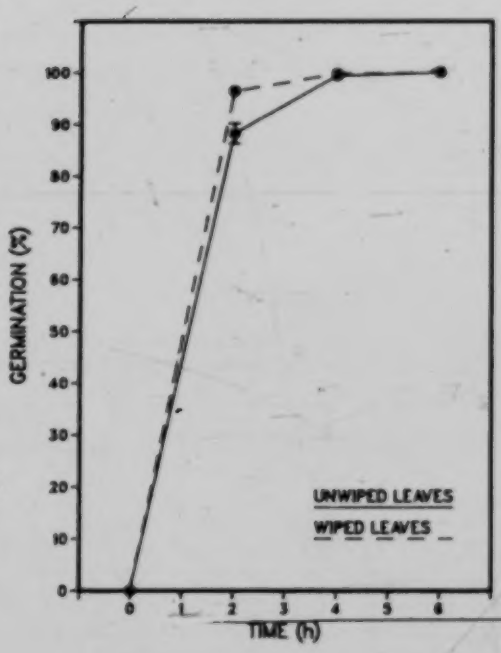
Effect of Leaf Epicuticular Wax on the Germination of the Conidia of A. brassicae.

Percent germination was determined two, four and six hours after inoculation.
The means are of five repeats and the bars represent standard error.

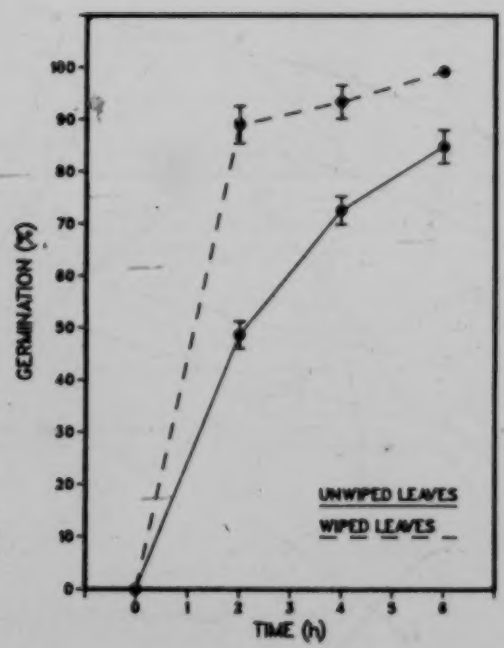
SLIDES



CANDLE



ALTEX



WESTAR

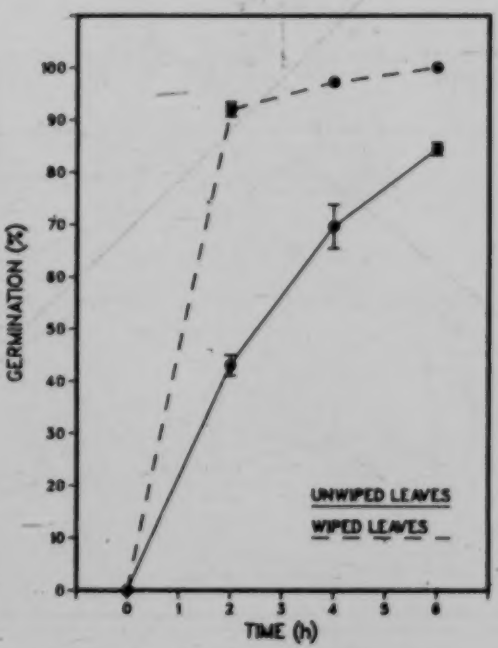
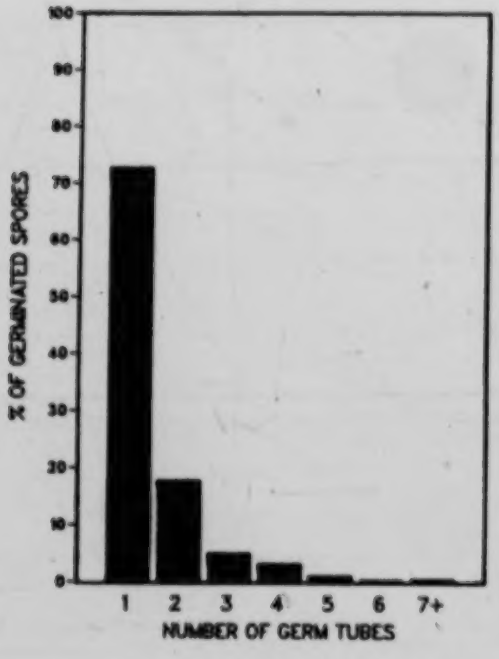


Figure 11a

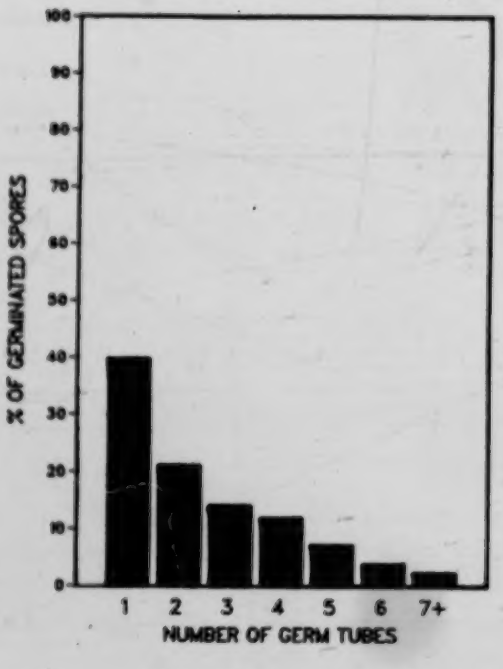
Effect of Leaf Epicuticular Wax on the Formation of Germ Tubes by the Conidia of A. brassicae.

Number of germ tubes were determined three hours after inoculation.
Each treatment is a summation of seven repeats.
Treatments were compared by Chi-square analysis (P values given in Figure 11c).
The seven germ tube groupings are expressed as a percent of the germinated spores.

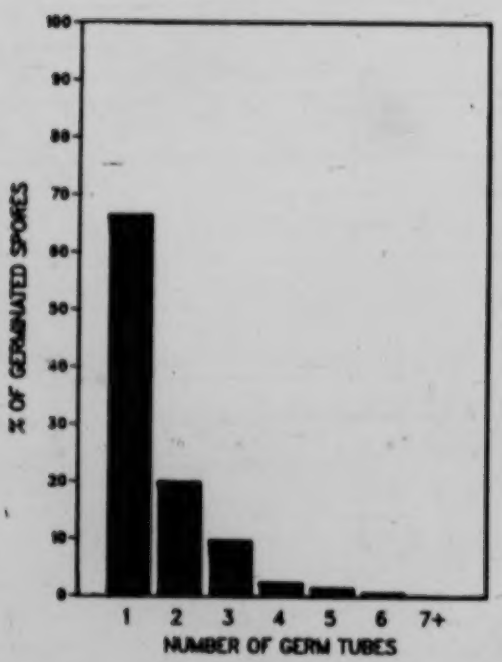
CANDLE(unwiped leaves)



CANDLE(wiped leaves)



TOBIN(unwiped leaves)



TOBIN(wiped leaves)

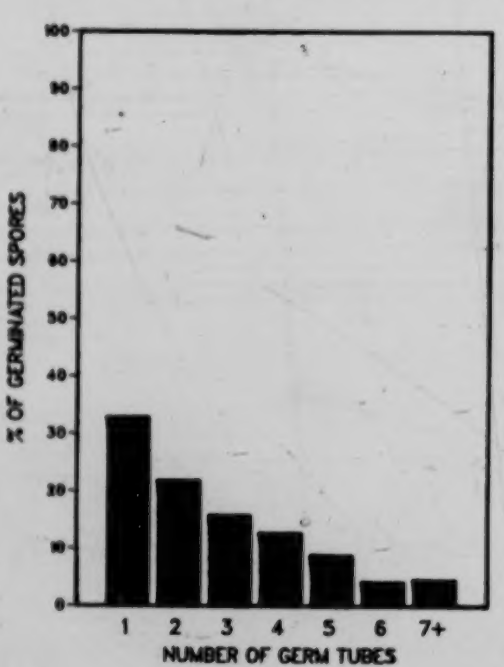
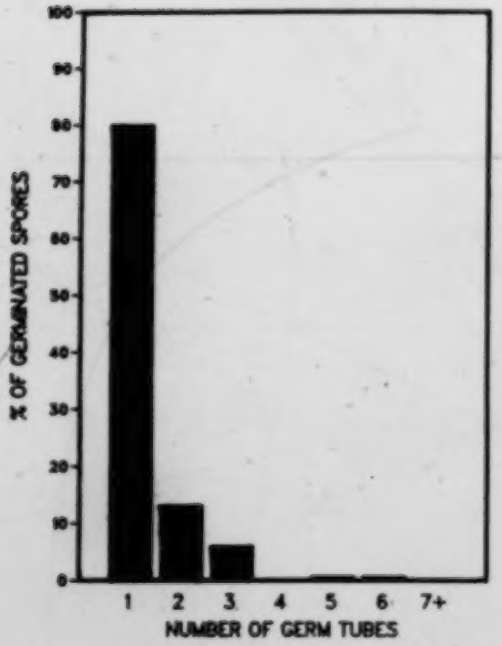


Figure 11b

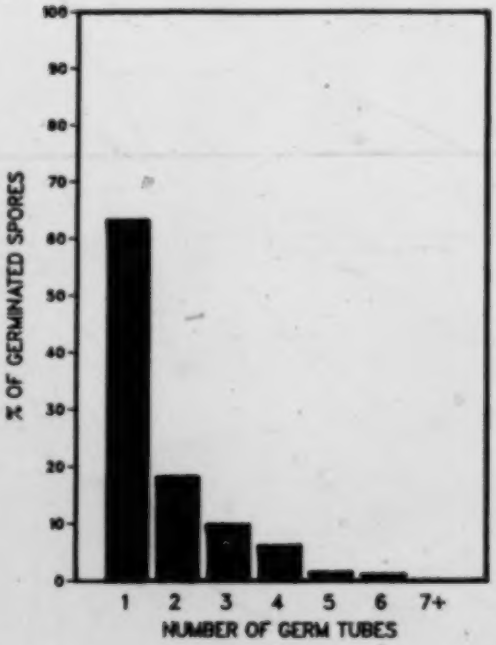
Effect of Leaf Epicuticular Wax on the Formation of Germ Tubes by the Conidia of A. brassicae.

This figure is a continuation of Figure 11a.

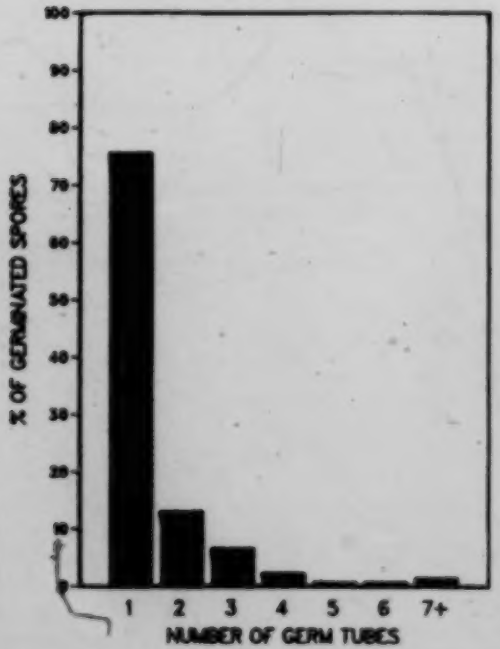
ALTEX(unwiped leaves)



ALTEX(wiped leaves)



WESTAR(unwiped leaves)



WESTAR(wiped leaves)

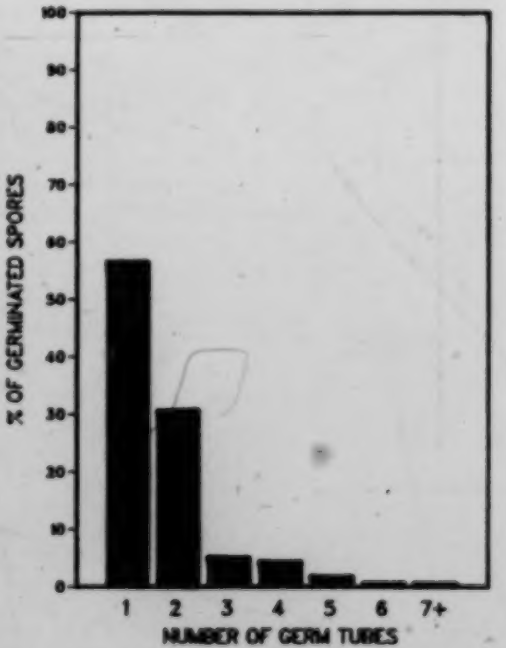


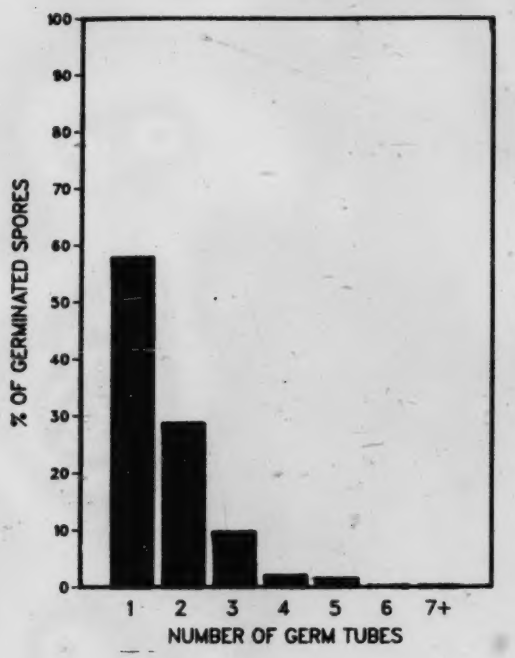
Figure 11c

Effect of Leaf Epicuticular Wax on the Formation of Germ Tubes by the Conidia of A. brassicae.

This figure is a continuation of Figures 11a and 11b. The P values listed here indicate the probability of two treatments being the same (Chi-square analysis).

Candle(C), Tobin(T), Altex(A), Westar(W) and Glass Slides.

SLIDES



	MEANS
C+	1.45
C−	2.47
T+	1.54
T−	2.80
A+	1.30
A−	1.69
W+	1.47
W−	1.69
SLIDE	2.06

		P
C+	C−	0.000
T+	T−	0.000
A+	A−	0.002
W+	W−	0.003
C+	T+	0.108
C−	T−	0.188
A+	W+	0.386
A−	W−	0.003

		P
SLIDE	C+	0.000
SLIDE	C−	0.000
SLIDE	T+	0.140
SLIDE	T−	0.000
SLIDE	A+	0.000
SLIDE	A−	0.001
SLIDE	W+	0.002
SLIDE	W−	0.143

\+ UNWIPED LEAVES
− WIPED LEAVES

Chapter III

Ultrastructure of Canola Epicuticular Wax

A. Introduction

All terrestrial organisms live under desiccating conditions and face the problem of water conservation. One measure that some organisms have developed to help combat this problem is the deposition of wax on their surface and/or as impregnation within their cuticle, providing a hydrophobic surface. Surface waxes can be found on higher plants, on some arthropods (e.g. insects and arachnids), even on some fungal spores and some bacteria (Caldicott and Eglinton, 1973; Hadley, 1981). This provides an example of parallel evolution in widely different groups of organisms. The biological properties of epicuticular waxes are dependent on their physical structure and chemical composition. This chapter deals with the former subject.

B. Materials and Methods

1. Plant Material

The cultivars of canola used were Tobin, Candle (B. campestris), Altex and Westar (B. napus). The plants were grown as described in chapter II. Middle leaves from positions five to seven and upper leaves from positions eight and nine were used for SEM. The adaxial leaf surfaces of all four cultivars were prepared for SEM by the two methods (A and B, described below). The abaxial leaf surfaces, stems and fruits of all four cultivars were prepared for SEM by method B. The stems from midway up of the plant and fruits from the lower, middle and upper parts of the inflorescence were used for SEM. Stems and fruits were examined because the blackspot lesions develop on these plant parts as well. The leaves were examined with SEM seven times and the stems and fruits twice. At each sampling two to three specimens were used.

2. Preparation of Plant Material for SEM

a) Method A: Freeze-drying

Leaves were placed in glass petri plates containing moist filter paper. Osmium tetroxide (1 ml of a 2% solution in 0.1M phosphate buffer, pH 7.0) was added to the filter paper, and the leaves fixed by osmium tetroxide vapor overnight. Pieces of the leaves (5 mm x 5 mm) were cut, frozen by quick immersion in liquid

Freon 22, stored in liquid nitrogen and freeze-dried at -80°C using an Edwards vacuum freeze-drier. The leaf pieces were then mounted on stubs with conductive glue, coated with gold and examined in a Cambridge Stereoscan 150 SEM.

b) Method B: Air-drying

Leaves were fixed with osmium tetroxide vapor as described above. Pieces of the leaves (1 cm x 1 cm) were cut, mounted on stubs and air-dried for two days. Pieces of the stems and fruits were placed on glass rods in petri plates containing moist filter paper and fixed with osmium tetroxide vapor as described above. Pieces of the stems and fruits, 1 cm long, were cut, mounted on stubs and air-dried for two days. All the materials were coated with gold and examined as described above.

C. Results and Discussion

1. Morphology of Canola Epicuticular Wax

The plant surfaces were examined with SEM at many different times during the course of this study. As some of the plants were grown in the greenhouse, the environmental conditions would not have been exactly the same each time. Therefore, some variation in the wax ultrastructure might be expected, because a complex epicuticular wax like that of Brassica is more sensitive to environmental factors than the less complex wax from other plants (Hunt et al., 1976; Jeffree et al., 1976). The density of the wax often varied from one sampling time to another. It was observed that higher temperatures and light intensities increased the "bloom" on the plants. This agrees with what has been reported by Armstrong and Whitecross (1976) and Whitecross and Armstrong (1972). Also, the size and proportion of each wax crystal type sometimes varied from surface to surface, and from one sampling time to another, but the same types of wax crystals were always present on all plant surfaces studied. No generalizations about this variation in ultrastructure could be made because the variables were not studied in detail.

SEM revealed that the leaves, stems and fruits of rapeseed were covered with a layer of wax crystals that comprise the "fluffy" layer of wax. These wax crystals were evenly distributed over the surface, except for the areas

immediately surrounding the stomata, where the wax was less dense. The top view of a Westar leaf (Fig. 13A) illustrates the distribution of the wax. When the plant surfaces were observed from an angle, the wax crystals could be seen in an oblique view. The oblique view revealed that many of the wax crystals project away from the plant surface, forming a "forest" of wax crystals (e.g. Westar leaf, Fig. 13B).

The leaves described above were prepared for SEM by the air-drying method (Method B). There was no apparent damage or disruption of the wax when this method was used. Such was not the case when the freeze-drying method (Method A) was used. Rapid immersion in molten Freon 22 is one of the steps involved in preparing specimens by the freeze-drying method. This results in brisk boiling of the coolant as heat is transferred from the specimen to Freon 22, perhaps resulting in physical disturbance in the wax layer. The SEM photographs of specimens prepared by the freeze-drying method indicated marked re-distribution and washing away of wax, leaving bare areas. The differences between the two methods of preparation can be seen by comparing freeze-dried leaves of Candle (Fig. 12C) and Westar (Fig. 13C) with their respective air-dried leaves (Figs. 12A and 13A). These results show that the freeze-drying method should not be used to study the ultrastructure of the wax. The use of this method would lead to incorrect interpretations of the epicuticular wax. It does not reveal the even distribution of the wax nor the correct orientation of the wax crystals

on the surface.

When studying an undisturbed leaf surface, one cannot discern whether there is a layer of amorphous wax present. There has been only a limited amount of evidence that the "fluffy" layer of wax is superimposed on a layer of amorphous wax. Evidence for the presence of an amorphous layer of wax was obtained from the disruption of the wax by the freeze-drying method. The micrograph (Fig. 21A) of a freeze-dried leaf shows a sheet of wax that has been turned upside down. It can be seen that the "fluffy" layer of wax crystals appears to be supported by a continuous layer of wax. The dislodged wax crystals in Figure 21B also appear to be connected by a layer of wax. Providing this evidence for the presence of an amorphous layer of wax was the only benefit derived from using the freeze-drying method.

The "fluffy" layer of wax appeared to be comprised of at least three types of wax crystals. These were plate-like crystals, filamentous, sometimes branched crystals, and rod-like crystals, present singly or forming blocks. The plates were oriented flat on the surface, while the rods and filamentous crystals projected away from the surface. The plates were of variable shape and size, and some contained holes (Fig. 21C). Some plates were barely discernable and others had well defined edges and were slightly raised. The rods were the most plentiful type of wax crystal. They appeared singly or in blocks. Individual rods were stocky and relatively straight (Fig. 22A). The wax crystals

described here as being blocks, appeared to be groups of rods fused together because their sides were undulating with contours corresponding to the individual rods. Most of the blocks contained one or more holes and they all had flat tops (Fig. 22B,C). The filamentous wax crystals were thinner and usually longer than the rods (Fig. 21D). They were also often curved and sometimes branched (Fig. 21E).

Wax crystals similar to the plates, filaments and individual rods described above have been reported before in rapeseed (Armstrong and Whitecross, 1976; Holloway et al., 1977; Tewari and Skoropad, 1976; Whitecross and Armstrong, 1972). However, this appears to be the first report of the occurrence of the wax crystals described here as blocks of fused rods. Rods might fuse together into a block because several sources of the wax components for rods were in close proximity, so close that the individual rods could not form. From the perspectives studied it appeared that fusion was initiated at the level of the amorphous layer of wax, and not above it (Fig. 22B,C). It did not appear that the blocks started as individual rods which later fused together. There was not one example found of two or more rods fused at the top and not at the bottom.

Most of the blocks when viewed from the top appeared similar to some of the wax crystals described as plates (Fig. 21C). The height of the blocks varied considerably. They ranged from tall ones to those that were just higher than some of the crystals described as plates (Figs. 22C,

23A,B). It is possible that some of the plate-like crystals are actually young blocks. Tall blocks have to develop from shorter blocks, and a very short block would look almost the same as some of the plates. This is not to say, however, that all plates eventually become blocks. There appeared to be growth rings in some of the blocks indicating periodic growth (Fig. 22C). The developmental sequences of rods and blocks of rods can be seen in Figure 23A,B. Also, there were always one or more holes in the blocks, but individual rods never had hollow centers.

One wax crystal type that was not found in this study was the hollow tube. Hollow tubes have been reported in some other cultivars of rapeseed (Tewari and Skoropad, 1976; Holloway et al., 1977). The blocks described here did contain holes, but were very different from the descriptions of hollow tubes, and thus were not likely related.

2. Density of Leaf Epicuticular Wax

With the naked eye, one could see that B. napus cultivars were glaucous and that B. campestris cultivars were not (Fig. 3). SEM showed that this was due to differences in the density of the wax crystals on the leaf surface, with Westar and Altex having the denser wax. This can be seen in the top views of the leaves (Candle, Fig. 12A; Westar, Fig. 13A; Tobin, Fig. 14A; Altex, Fig. 15A). The oblique views of the wax crystals can be seen in Figures 12B, 13B, 14B and 15B for Candle, Westar, Tobin and Altex,

respectively. This means that the glaucousness of B. napus as compared to the condition in B. campestris is due to the density of the wax crystals, and not to the presence of different types of crystals.

There was not much difference in the density of the wax crystals between middle and upper leaves of B. napus cultivars (Fig. 15B,C). Brassica campestris cultivars, however, differed in this respect. The upper leaves of B. campestris cultivars had a more dense layer of wax than the middle leaves (Fig. 14B,C). This indicated that young leaves of B. campestris were capable of producing a substantial amount of wax, but when they became older, wax production was greatly reduced. This also indicated that B. campestris leaves have the genes to produce larger amounts of wax, but are somehow switched off from doing so as the leaves age.

So far, only the adaxial leaf surfaces have been described. The abaxial leaf surfaces were also examined by SEM. There was no difference in the types of wax crystals present on the two surfaces. This is evident from views of the adaxial (Fig. 15B,C) and abaxial (Fig. 16A,B) leaf surfaces of Altex. There was also not much difference in the density of wax crystals on the two leaf surfaces of Altex. However, not enough abaxial leaf surfaces were examined to make any generalized statements about the density of the wax between the adaxial and abaxial leaf surfaces.

3. Density of Stem Epicuticular Wax

The stems of the four cultivars were also compared. The types of wax crystals present on the stems were similar to those on the leaves (Figs. 17A,B - Westar; 18A,B - Candle; 19A,B - Altex; 20A,B - Tobin). Unlike the leaves, there was no appreciable difference in the density of the wax crystals among cultivars. The density of wax crystals was high for all the cultivars (Figs. 17A,B - Westar; 18A,B - Candle; 19A,B - Altex; 20A,B - Tobin). The wax on all the stems was as dense or denser than that found on any leaf examined. The high density of wax crystals on the stems of the B. campestris cultivars (Figs. 18A,B - Candle; 20A,B - Tobin) again indicated their ability to produce large amounts of wax.

4. Density of Fruit Epicuticular Wax

The fruits of the four cultivars were compared. The density of wax crystals was similar to that on the leaves. The fruits of Altex (Fig. 19C) and Westar (Fig. 17C) had a heavier layer of wax than those of Candle (Fig. 18C) and Tobin (Fig. 20C). The wax crystals on the surface of Candle and Tobin fruits were as dense as those on Altex and Westar, but were shorter and stubbier. Fruits from the lower, middle and upper parts of the inflorescence were compared. There was no difference in the wax between a very young fruit and an older, much larger fruit. This can be seen by comparing an older fruit of Westar (Fig. 17C) to a younger

fruit (Fig. 16C). This indicated that the wax was formed on the surface very early and did not change much thereafter.

D. Conclusions

The air-drying method of preparing plant surfaces for SEM was better than the freeze-drying method. The surface of canola was covered with an evenly distributed layer of wax crystals superimposed on an amorphous layer of wax. The density of the wax crystals varied from cultivar to cultivar and from one part of the plant to another, but all the plant surfaces had the same types of wax crystals. Some trends such as the density of the wax on the leaves and fruits appeared to be species specific, whereas the density of the wax on the stems did not. There appeared to be at least three types of wax crystals present. These included plate-like crystals, filamentous, sometimes branched crystals, and rods, present singly or forming blocks. The blocks of rods have been observed in the cultivars examined for the first time in rapeseed.

E. Figures and Legends

Figure 12

A. Top view of the adaxial surface of a middle leaf of Candle, prepared for SEM by method B. Note the even distribution of wax crystals (magnification x1,800).

B. Oblique view of the adaxial surface of a middle leaf of Candle, prepared for SEM by method B. Note that most of the wax crystals project away from the surface (magnification x5,000).

C. Top view of the adaxial surface of an upper leaf of Candle, prepared for SEM by method A. Note the displacement of wax crystals, leaving bare areas (magnification x2,000).

a
b
c

Figure 13

A. Top view of the adaxial surface of a middle leaf of Westar, prepared for SEM by method B. Note the even distribution of wax crystals (magnification x1,800).

B. Oblique view of the adaxial surface of a middle leaf of Westar, prepared for SEM by method B. Note that most of the wax crystals project away from the surface (magnification x5,000).

C. Top view of the adaxial surface of an upper leaf of Westar, prepared for SEM by method A. Note the displacement of wax crystals leaving bare areas (magnification x1,100).

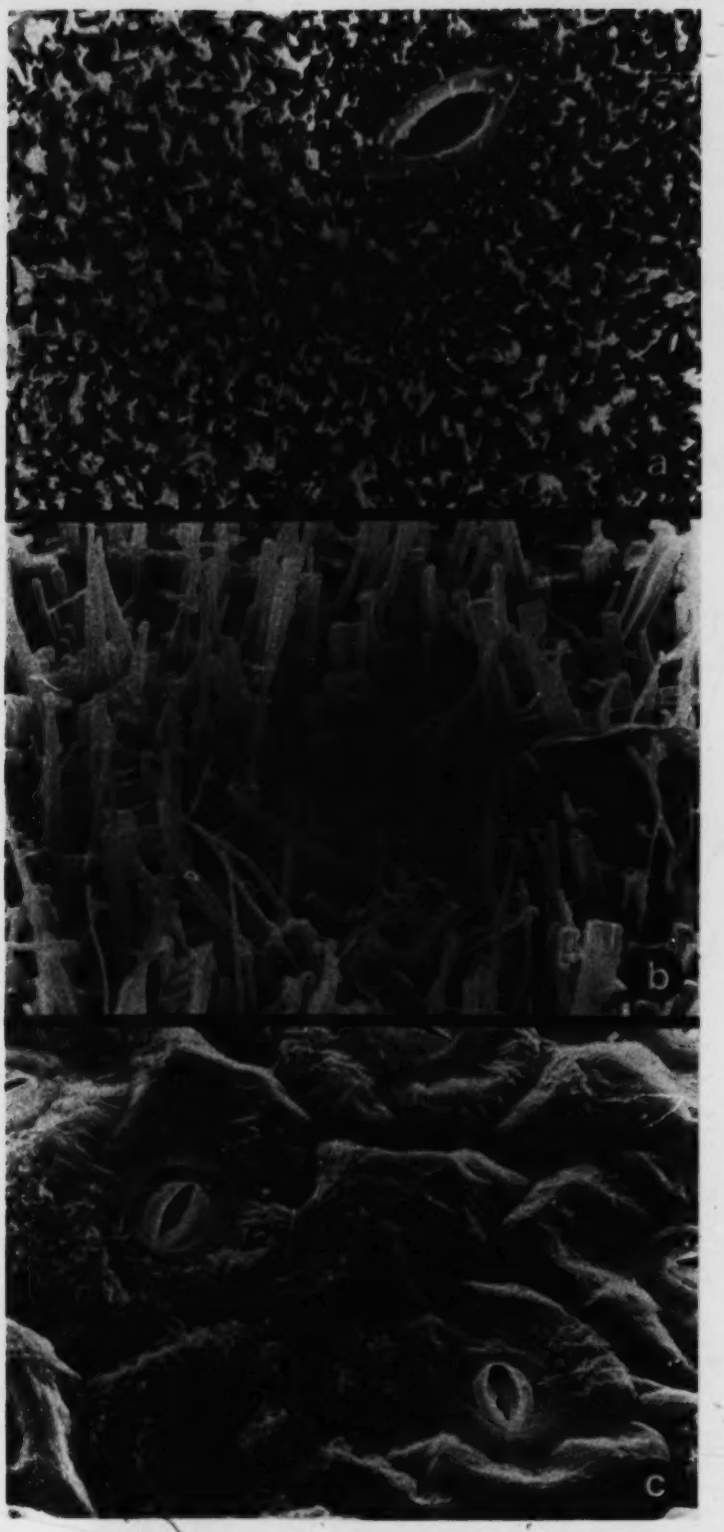

a
b
c

Figure 14

A. Top view of the adaxial surface of a middle leaf of Tobin. Note the even distribution of wax crystals (magnification x1,800).

B. Oblique view of the adaxial surface of a middle leaf of Tobin (magnification x5,000).

C. Oblique view of the adaxial surface of an upper leaf of Tobin. Note the higher density of wax than on the leaf from the middle of the plant (B) (magnification x5,000).

a
b
c

Figure 15

A. Top view of the adaxial surface of a middle leaf of Altex. Note the even distribution of wax crystals (magnification x1,800).

B. Oblique view of the adaxial surface of a middle leaf of Altex (magnification x5,000).

C. Oblique view of the adaxial surface of an upper leaf of Altex. Note that there was not much difference in the density of the wax between middle (B) and upper leaves (magnification x5,000).

C

Figure 16

A. Top view of the abaxial surface of an upper leaf of Altex (magnification x2,600).

B. Oblique view of the abaxial surface of an upper leaf of Altex. Note that the surface appears to be similar to the adaxial leaf surface (Fig. 15B) (magnification x6,500).

C. Oblique view of the fruit surface of Westar from the upper part of the inflorescence (magnification x9,000).

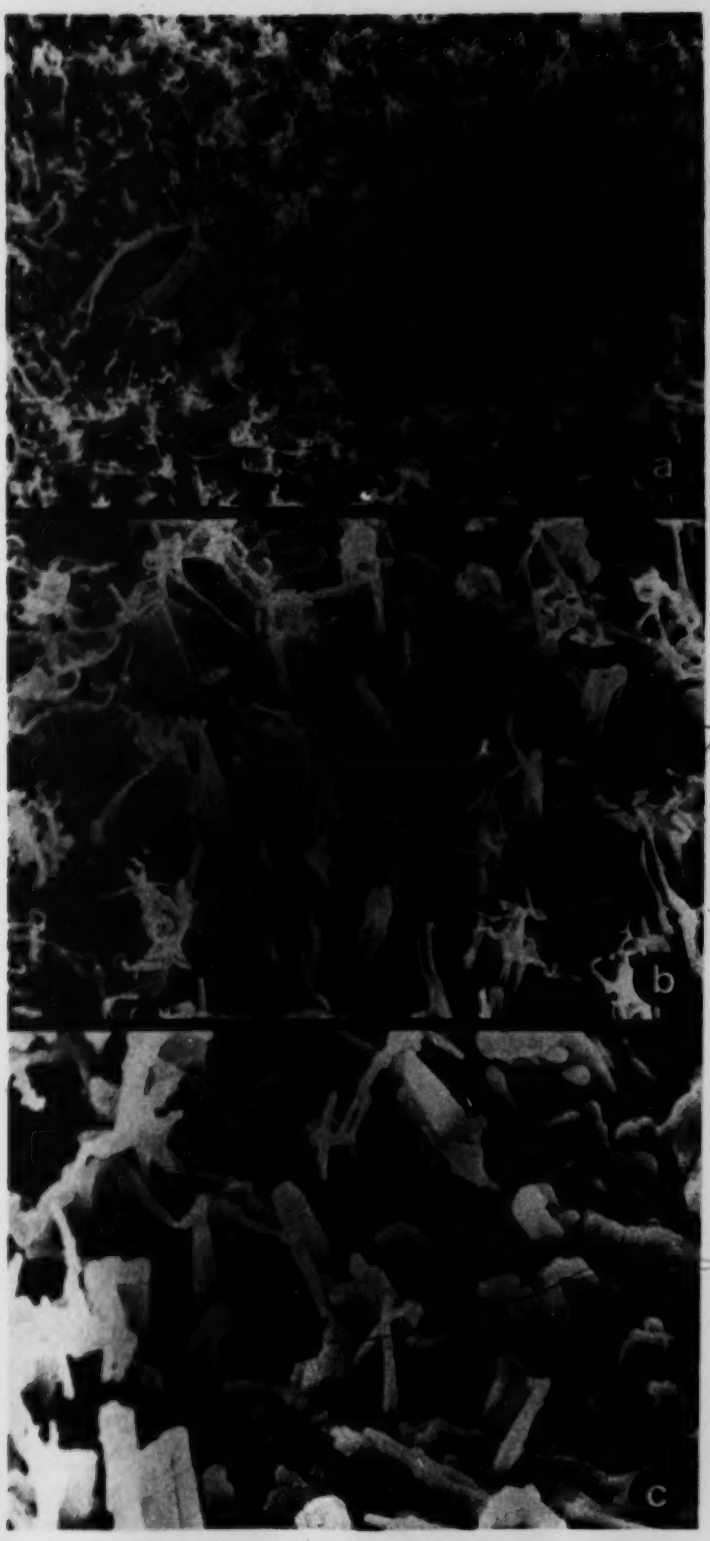

a
b
c

Figure 17

A. Top view of the stem surface of Westar from the middle of the plant. Note the even distribution of wax crystals (magnification x1,600).

B. Oblique view of the stem surface of Westar from the middle of the plant. Note the high density of wax crystals (magnification x6,000).

C. Oblique view of the fruit surface of Westar from the lower part of the inflorescence. Note that this surface appears to be similar to that of the fruit from the upper part of the inflorescence (Fig. 16C) (magnification x4,500).

b
c

Figure 18

A. Top view of the stem surface of Candle from the middle of the plant. Note the even distribution of wax crystals (magnification x1,600).

B. Oblique view of the stem surface of Candle from the middle of the plant. Note the high density of wax crystals (magnification x6,000).

C. Oblique view of the fruit surface of Candle from the middle of the inflorescence. Note the short wax crystals (magnification x4,500).

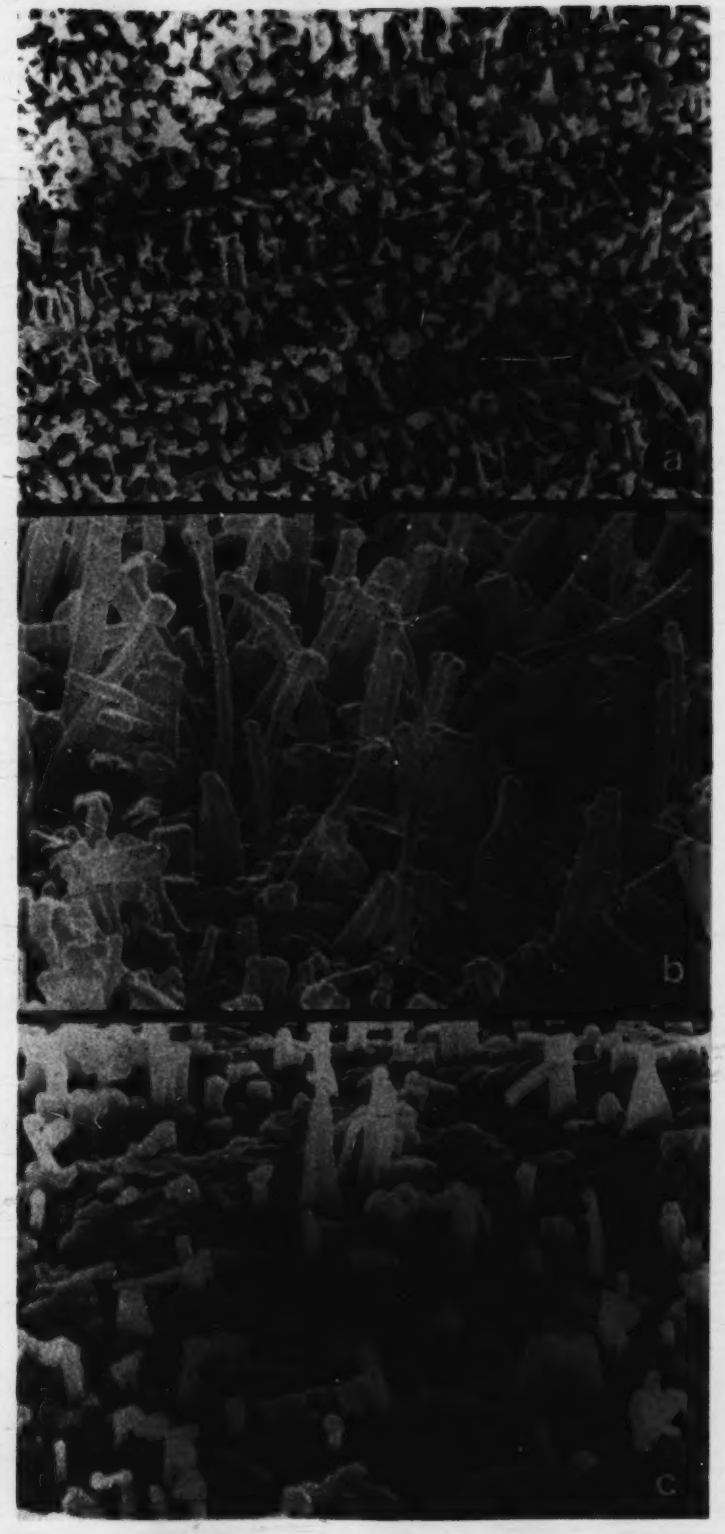

a
b
c

Figure 19

A. Top view of the stem surface of Altex from the middle of the plant. Note the even distribution of wax crystals (magnification x1,600).

B. Oblique view of the stem surface of Altex from the middle of the plant. Note the high density of wax crystals (magnification x5,000).

C. Oblique view of the fruit surface of Altex from the upper part of the inflorescence (magnification x5,500).

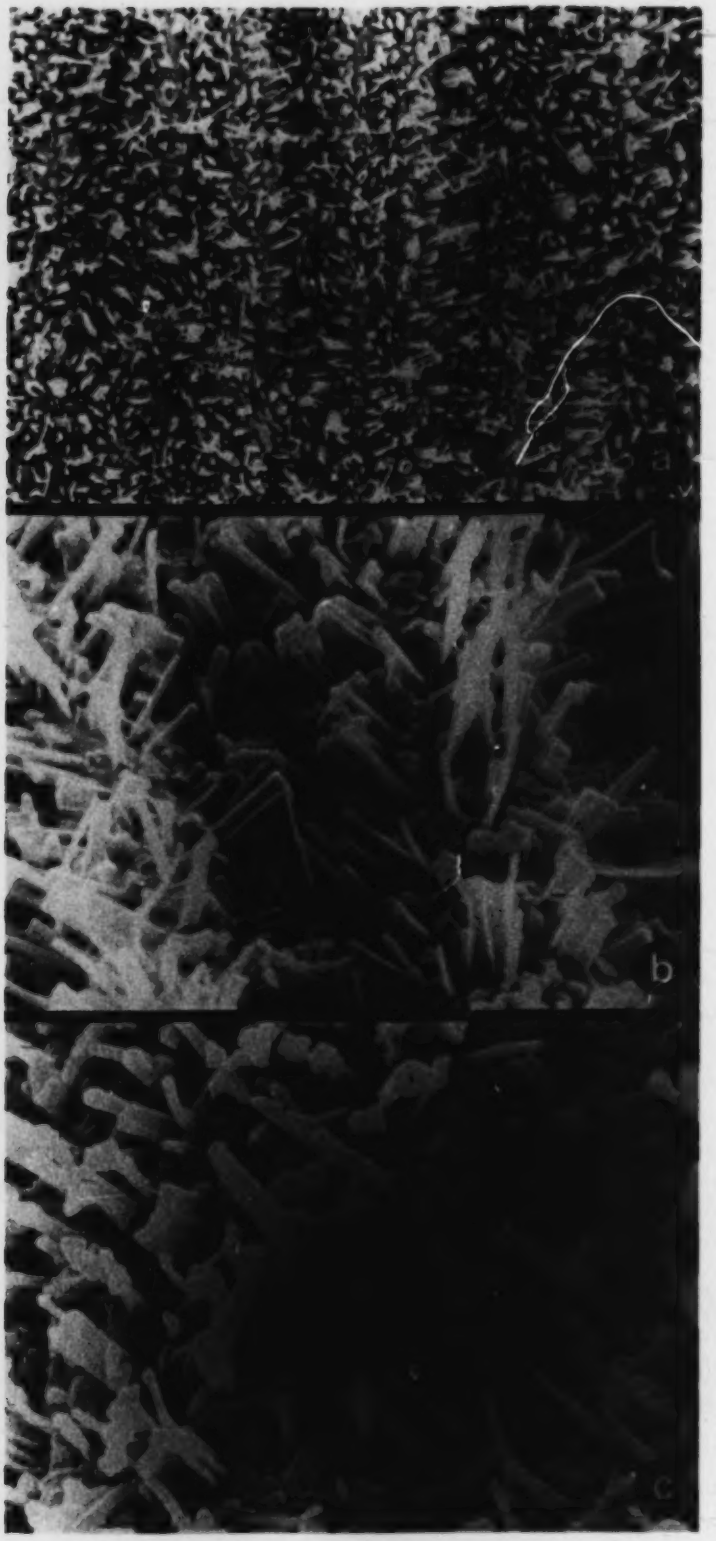

a
b
c

Figure 20

A. Top view of the stem surface of Tobin from the middle of the plant. Note the even distribution of wax crystals (magnification x1,400).

B. Oblique view of the stem surface of Tobin from the middle of the plant. Note the high density of wax crystals (magnification x6,000).

C. Oblique view of the fruit surface of Tobin from the upper part of the inflorescence. Note the short wax crystals (magnification x2,500).

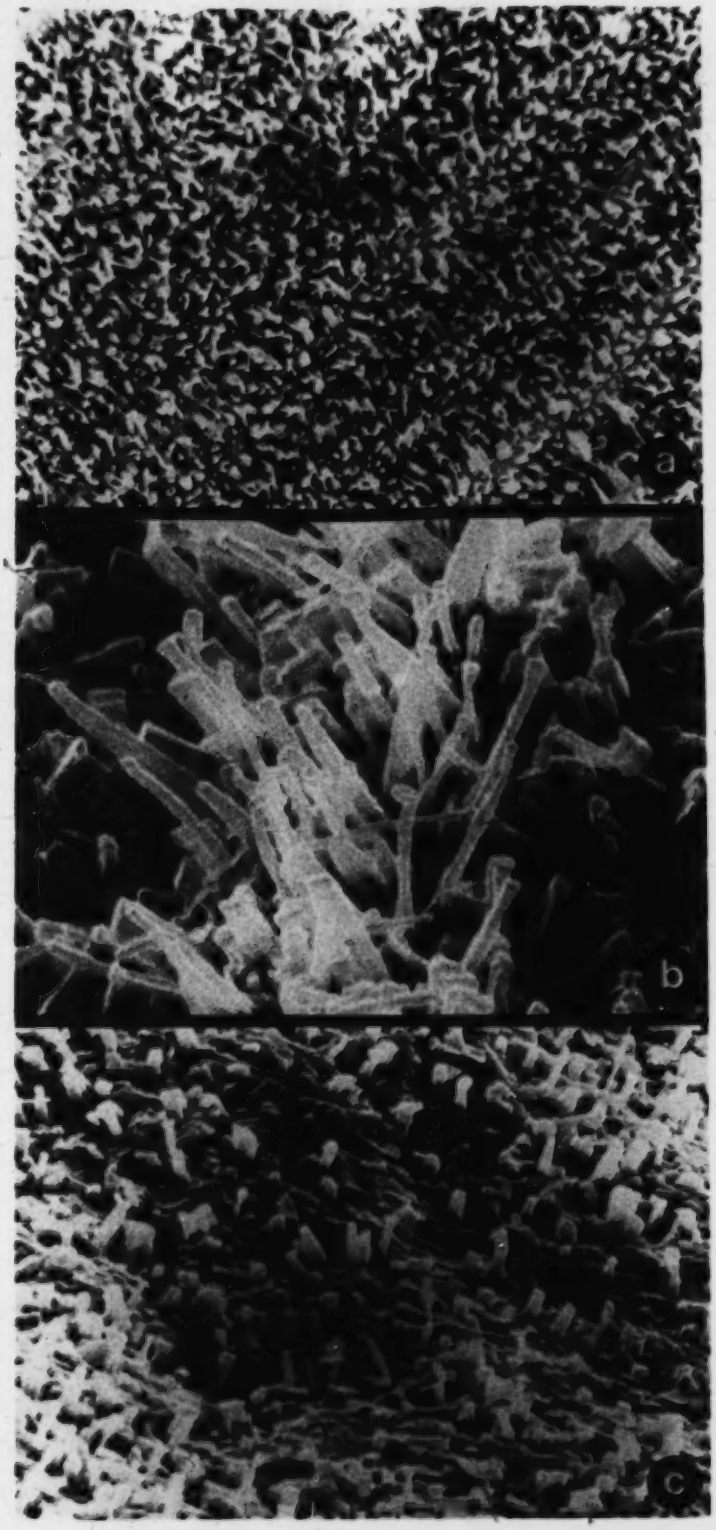

a
b
c

Figure 21

A. Adaxial surface of an upper leaf of Westar prepared for SEM by method A, showing a sheet of wax that has been turned upside down (magnification x1,800).

B. Adaxial surface of an upper leaf of Westar prepared for SEM by method A, showing dislodged wax crystals (magnification x4,500).

C. Adaxial surface of an upper leaf of Altex showing plate-like wax crystals (arrows) (magnification x7,000).

D. Adaxial surface of an upper leaf of Tobin showing filamentous wax crystals (arrows) (magnification x8,000).

E. Adaxial surface of a middle leaf of Tobin showing a branched filamentous wax crystal (arrow) (magnification x7,700).

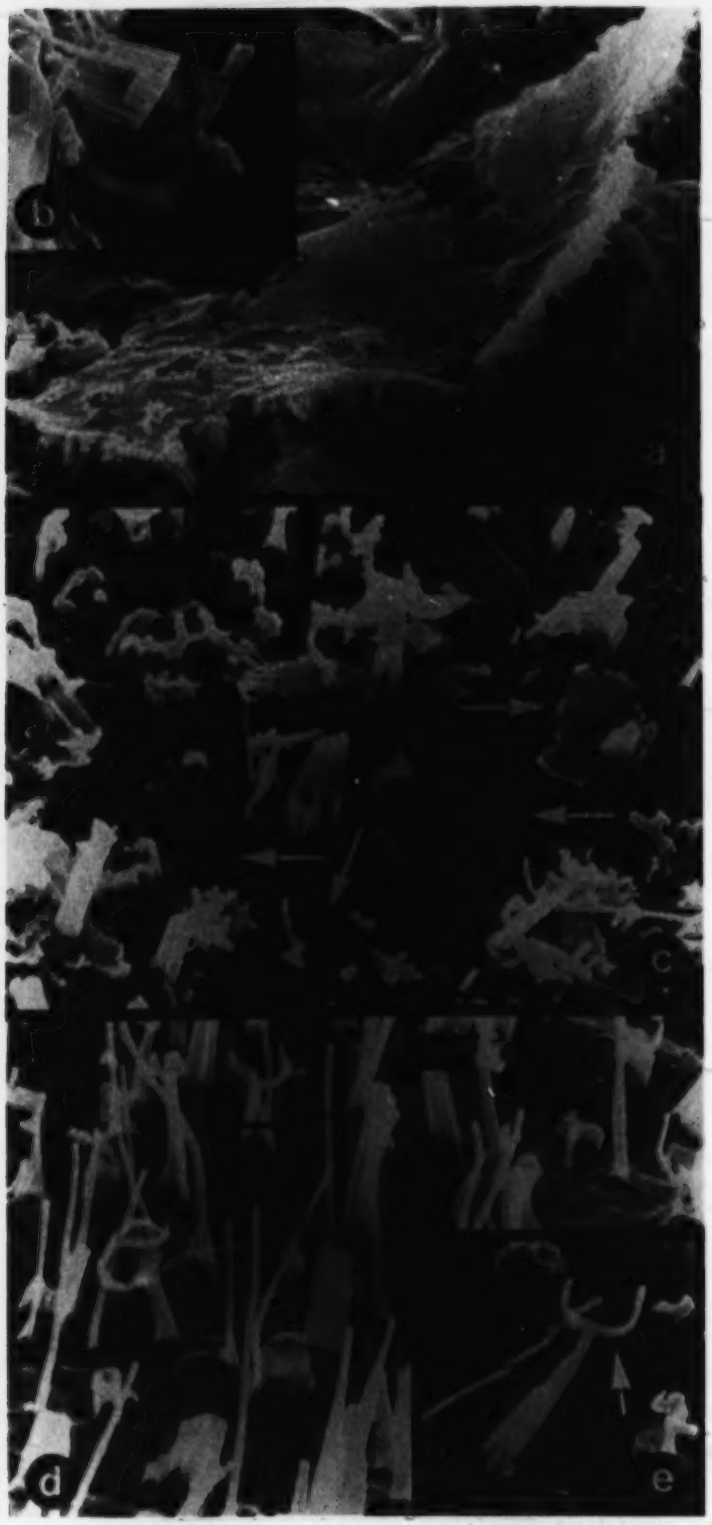

b
c
d
e

Figure 22

A. Stem surface of Altex from the middle of a plant showing rods (magnification x12,100).

B. Abaxial surface of an upper leaf of Tobin showing a fused rod (arrow) (magnification x18,000).

C. Adaxial surface of an upper leaf of Westar showing fused rods (arrows). Note the growth rings in these crystals (magnification x22,600).

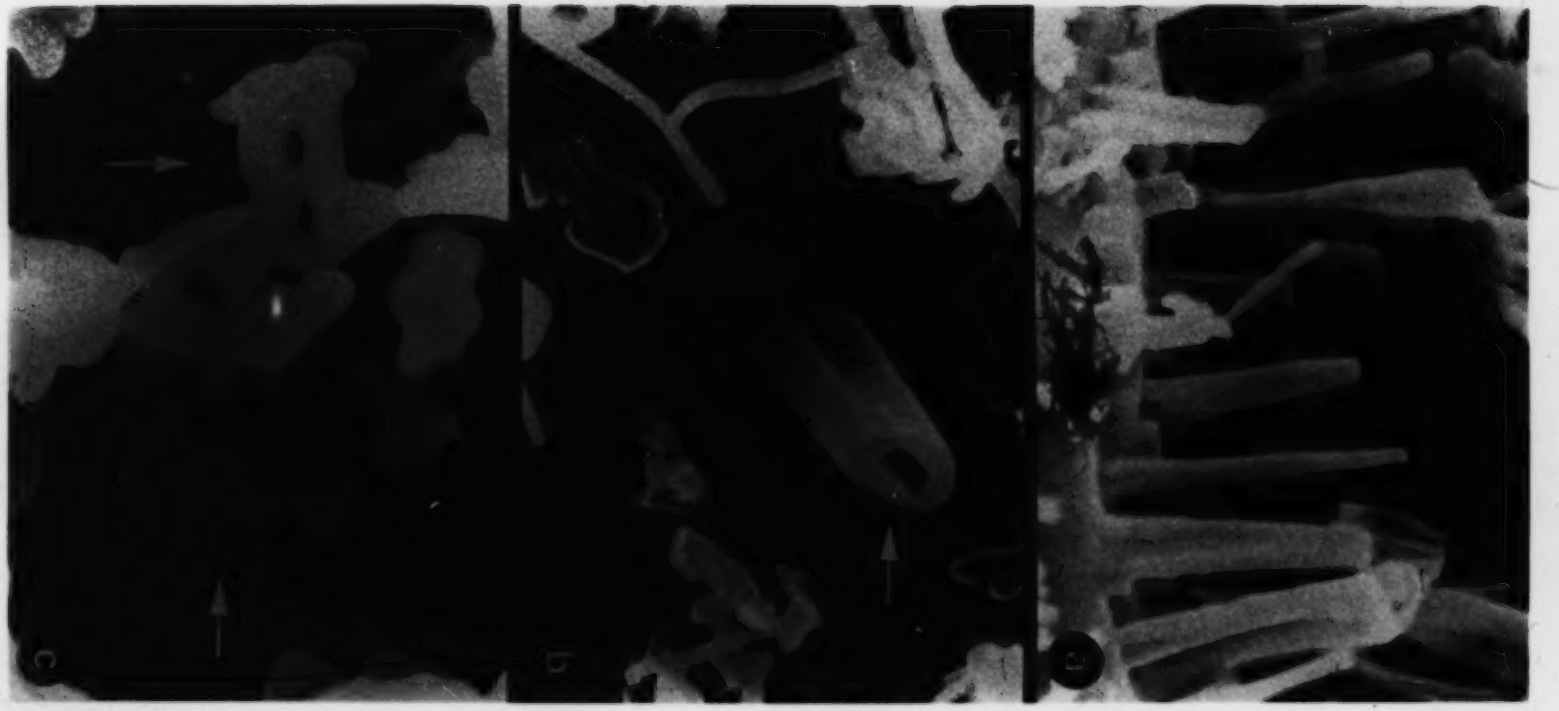

c
d
e

Figure 23

A. Stem surface of Westar from the middle of the plant showing plate-like crystals and fused rods (note arrows) (magnification x16,000).

B. Stem surface of Altex from the middle of the plant showing the wax crystals at different stages of development (arrows) (magnification x15,500).

Chapter IV

Chemistry of Canola Leaf Epicuticular Wax

A. Introduction

It has been demonstrated that the epicuticular wax is one of the factors involved in the differential susceptibility of B. napus and B. campestris to A. brassicae. The biological properties of epicuticular waxes are dependent on their physical structure and chemical composition, and the ultrastructure of the wax is principally determined by its composition (Baker, 1982). The previous chapter dealt with the ultrastructure of wax in the cultivars of B. napus and B. campestris. The chemistry of epicuticular waxes in rapeseed has so far been studied only in three Europeon strains of B. napus (Holloway et al., 1977). This chapter reports on the chemistry of waxes in four Canadian cultivars of B. napus and B. campestris.

B. Materials and Methods

1. Plant Material

The cultivars of canola used were Tobin, Candle (B. campestris), Altex and Westar (B. napus). The plants were grown as described in chapter II. Since large numbers of leaves were required for wax extraction, all the leaves from positions two to eight were used.

2. Extraction of Wax

The wax was extracted from the leaf surfaces by immersing the leaves individually for two to three seconds in 400 ml chloroform at room temperature with gentle agitation. The chloroform extract was then filtered through a Whatman #1 filter paper applying vacuum (water pump), evaporated overnight in a fume hood, and the wax collected and weighed. The surface area of the leaves was determined by tracing the leaves on paper, cutting the tracings out, and comparing the weight of the tracings to the weight of a known area of paper.

3. Fractionation of Wax

a) Thin Layer Chromatography

Thin layer chromatography (TLC) was used to separate the wax from the four cultivars into different classes of constituents. Silica gel 60G (Merck) was used as the adsorbent to make plates 0.25 mm thick using

a Desaga Heidelberg adsorbent spreader. 25 g of silica gel in 60 ml of water were sufficient to make five plates (20 cm x 20 cm). The silica was activated by heating the plates at 110°C for two hours. The wax was dissolved in chloroform (20 mg/ml), and 250 µl of this solution (5 mg of wax) was spotted over a 16 cm length of each plate. The plates were then developed in benzene:hexane:chloroform (8:1:1), allowing the solvent front to move 14 cm. Some other solvent systems were also tried, but they did not give as good a separation of the bands. These included benzene (Holloway et al., 1977; Flore and Bukovac, 1978), benzene:chloroform (7:3) (Freeman et al., 1979), hexane and benzene:hexane (9:1). After the plates were developed they were sprayed with 50% sulfuric acid in water (w/w) (Bukovac et al., 1979) and heated at 150°C for 15 minutes resulting in charring of the bands. The plates were also observed under UV light to help visualize the bands. A 0.5% aqueous solution of the indicator Rhodamine 6G (Freeman et al., 1979a; Leece, 1976) was also tried, but it did not show the bands as well as sulfuric acid.

b) Semi-Preparative Layer Chromatography

Semi-preparative layer chromatography (Semi-PLC) was used to quantitatively separate the wax from Altex into different classes of constituents. Silica gel plates 0.25 mm thick were prepared as previously described and used for Semi-PLC. This technique was

called Semi-PLC because preparative layer chromatography (PLC) usually involves plates of at least 1 mm thickness. 25 mg of wax per 1 mm thick plates was tried; however, the separation of the bands was not as good as in the thinner plates, so only 0.25 mm thick plates were used. The wax was spotted on the plates, and the plates developed as described above. After the plates were developed, the vertical edges of each plate were sprayed with 50% sulfuric acid in water (w/w) and heated at 75°C for 30-40 minutes resulting in slight charring of some of the bands. The temperature used here was lower than that used for TLC, because the bands were to be further analysed, and the higher temperature might have altered the wax fractions. The plates were then observed under UV light to outline the bands, using the sprayed vertical edges as a guide. In addition to this, some of the bands in the unsprayed middle portions could be seen as darker areas, which helped in outlining them. The bands from the unsprayed middle portions of 20 plates were scraped off into separate glass columns (1 cm x 20 cm). Anhydrous ethyl ether (Et_2O, 50 ml) was passed through each column and collected in round bottom flasks (Holloway and Brown, 1977). The ethyl ether was then evaporated using a flash evaporator, and the fractions analysed by TLC.

c) Column Chromatography

Separation of the wax from the cultivar Altex was also done using a silicic acid column based on the work done by Tulloch and Hoffman (1974). Silicic acid (Biosil A, 100-200 mesh, 100 g) was used in a 30 cm x 3.5 cm column. 1 g of wax dissolved in hexane (50 ml) was loaded on the column. The following series of solvent systems, from non-polar to polar, were then passed through the column:

hexane (500 ml)
hexane:Et_2O (99:1, 1 l)
hexane:Et_2O (99:1, 2.5 l)
hexane:Et_2O (98:2, 1 l)
hexane:Et_2O (97:3, 1.5 l)
hexane:Et_2O (96:4, 1.5 l)
hexane:Et_2O (96:4, 3.5 l)
hexane:Et_2O (93:7, 1 l)
hexane:Et_2O (92:8, 1.5 l)
hexane:Et_2O:ethanol (70:25:5, 1.5 l)

The eluent fractions were collected and concentrated using a redistillation apparatus. The fractions were then analysed by TLC.

4. Infrared Spectroscopy

Infrared spectroscopy (IR) was carried out for the fractionated Altex wax obtained by Semi-PLC, using a Nicolet 7199 FTIR spectrometer.

5. Gas-Liquid Chromatography

The waxes from the four cultivars and some of the component classes of the wax from Altex obtained from Semi-PLC, were dissolved in chloroform and analysed by gas-liquid chromatography (GLC). 1 µl (10 µg) of each sample was chromatographed using a Hewlett Packard Model 5830H gas chromatograph. Flow rate of the helium carrier gas was 50 ml/min. The chromatograph was fitted with a 3 mm x 1.2 m stainless steel column containing 1% Dexil 300 (Holloway et al., 1977). The oven temperature was programmed to increase from 120°C to 330°C at a rate of 3°C/min, based on the work done by Bukovac et al. (1979), Cowlishaw et al. (1983), Flore and Bukovac (1978), Macey and Barber (1970) and Tulloch (1983). The injection port and the hydrogen flame ionization detector temperatures were 325°C and 330°C, respectively. Identification of n-alkane peaks was done by comparison of peak retention times with retention times of the known hydrocarbon analytical standards (Analabs-New England Nuclear; PolyScience Corporation).

C. Results and Discussion

1. Amount of Epicuticular Wax on Canola Leaves

A two to three second extraction of the leaves in chloroform was used, which was shorter than those reported in the literature. This treatment appeared to be sufficient to remove the epicuticular wax. After drying, the extracted wax had a clean white color. If the leaves were dipped in chloroform much longer, the resulting wax had a greenish tinge. This was especially true for the B. campestris cultivars. This indicated that an extraction time of more than about three seconds resulted in the extraction of more than just the epicuticular wax. Table 3 shows the amount of wax on the leaf surfaces of the four cultivars of canola. This is an average for both surfaces of the leaves from positions two to eight, sampled two to three times, with an average sample of 2600 cm^2 of leaf area. The two cultivars of B. campestris (Candle and Tobin) showed similar amounts

Table 3

Amounts of Wax on the Leaves of Canola.

Cultivar	Wax ($\mu g/cm^2$)
Candle	43
Tobin	39
Altex	92
Westar	104

of wax and so did those of B. napus (Altex and Westar) as well. The B. napus cultivars had more than twice as much wax as the B. campestris cultivars. This indicates that the presence of two to three times the amount of wax on the B. napus cultivars relative to that in B. campestris is enough to confer lower susceptibility to A. brassicae.

The relative amounts of wax on the leaves of different plant species can vary considerably, ranging from a few $\mu g/cm^2$ to several 100 $\mu g/cm^2$ (Baker, 1982).

2. Chemistry of Canola Leaf Epicuticular Wax

a) Major Classes of Constituents

The extracted wax from the four cultivars was separated into nine bands on TLC plates (Fig. 24). These bands represented nine different classes of constituents, which have been identified from IR spectra (Appendix 1) and from comparison with data presented by Bukovac et al. (1979), Flore and Bukovac (1978), Holloway et al. (1977) and Knowles and Flore (1983). In the order of elution on TLC plates, the classes of constituents included alkanes, esters, ketones, aldehydes, sec-alcohols, ketols, prim-alcohols, triterpenols and fatty acids (Fig. 24). Figure 24 shows the waxes from the four cultivars separated by TLC, sprayed with 50% sulfuric acid and heated at 150°C for 15 minutes. It can be seen that all four cultivars contained the same bands, each with similar density

(Fig. 24). In this photograph the sixth and eighth bands are faint, but can be easily seen under UV light. When TLC plates were heated at 75°C for 30-40 minutes, the bands were not charred as much, and showed up as colored bands under UV light (Fig. 24).

Thus, all four cultivars contained the same nine classes of constituents. Holloway et al. (1977) also reported the same nine classes in three lines of B. napus (Nilla, a Nilla mutant and a Rigo mutant). Flore and Bukovac (1978) and Knowles and Flore (1983) reported all these classes of compounds in the wax of cabbage except for the triterpenols. The percent composition of each class was determined for Altex using Semi-PLC (Table 4). It can be seen that the alkanes make up almost 50% of the total wax. The esters and sec-alcohols are the next most abundant constituents.

b) Major Constituents

The waxes from the four cultivars and some of the component classes of the wax from Altex were examined by GLC in order to obtain information on the major constituents present. The general pattern of wax chain lengths was very similar for the four cultivars (Figs. 25-28). The major constituents were C_{29} alkane, C_{29} ketone, C_{29} sec-alcohol and C_{44}-C_{44} esters (Figs. 25-28). These GLC-tracings matched closely with those reported for cabbage (Flore and Bukovac, 1978) and for rapeseed (Holloway et al., 1977). The C_{29} alkane peak

Table 4

Percent Composition of the Major Wax Classes from the Leaf Epicuticular Wax of Altex.

Component Class	(%)*
Alkanes	48.5
Esters	16.3
Ketones	4.9
Aldehydes	1.6
Sec-Alcohols	12.9
Ketols	1.6
Prim-Alcohols	7.3
Triterpenols	5.1
Fatty Acids	1.9

* Percent composition determined from total wax recovered from Semi-PLC.

on these GLC-tracings was identified by comparison with the GLC-trace of the alkane fraction (Fig. 29), which was identified by comparison with an n-alkane standard. C_{29} alkane was the major alkane present. Figure 29 reveals that other alkanes were also present in small amounts, such as C_{25}, C_{27} and C_{31}, as identified with the help of n-alkane standards. The esters (C_{40}-C_{48}) were identified by comparison with the GLC-trace of the ester fraction (Fig. 30) and based on the data reported by Flore and Bukovac (1978). The GLC-trace of the ester fraction also showed some C_{29} alkane and C_{29} ketone. These were also demonstrated by TLC. No other wax fractions besides the esters had carbon-chain lengths in the range of C_{40}-C_{48}. The C_{29} ketone peak was identified by comparison with the GLC-trace of the ketone fraction (Fig. 31) and from data reported by

Flore and Bukovac (1978). Figure 31 shows that C_{31} ketone comprised almost all of the ketone class. The C_{29} sec-alcohol peak was identified from the GLC-trace of the sec-alcohol fraction (Fig. 32) and from data reported by Flore and Bukovac (1978). This was the major sec-alcohol present, but Figure 32 also shows the presence of some smaller carbon-chain sec-alcohols. Figure 33 shows the GLC-trace for the aldehyde fraction. It can be seen that the aldehyde class was composed of many constituents, with no one dominant constituent (Fig. 33). Figure 34 shows the GLC-trace for the prim-alcohol fraction which showed three to four major constituents. Holloway et al. (1977) reported that the major prim-alcohols in the rapeseed lines they studied were C_{24}-C_{28}. They also reported that the major triterpenols in those lines were α-amyrin and β-amyrin, and that the fatty acids were in the range of C_{14}-C_{20}. The triterpenol fraction from Altex was not examined by GLC, because no standards were available for comparison. The fatty acid fraction was not examined, because derivatives of the fatty acids would have had to be made and different GLC conditions developed to analyse them.

The separation of wax into different classes of constituents by the use of column chromatography was not as successful as that by using Semi-PLC. The fractions collected did not contain individual classes of constituents; instead, they usually contained two or

three classes of constituents. This procedure could be a good initial step in fractionation of wax, to be followed by PLC, because a large amount of wax can be partially separated. Subsequent use of PLC would not involve the separation of all the nine classes at once. This could be very useful for further studies that may require larger amounts of wax.

D. Conclusions

The chemical composition of epicuticular waxes is reported for the first time for the Canadian cultivars of canola. This is also the first study of its kind for *B. campestris*.

A short three second extraction in chloroform appeared to be sufficient to remove the leaf epicuticular wax of canola. This indicated that some wax extractions reported in the literature may have been too vigorous for removal of just the epicuticular wax. The amount of epicuticular wax on *B. napus* leaves was over twice that on the *B. campestris* leaves.

The general chemical composition of the leaf epicuticular waxes was very similar in all four cultivars studied. Thus, wax chemistry does not seem to be responsible for the differences in susceptibility of *B. napus* and *B. campestris* to *A. brassicae*. The waxes consisted of nine major classes of constituents, which included alkanes, esters, ketones, aldehydes, sec-alcohols, ketols, prim-alcohols, triterpenols and fatty acids. The major constituents of the waxes were C_{31} alkane, C_{29} ketone, C_{29} sec-alcohol and C_{44}-C_{46} esters.

E. Figures and Legends

Figure 24

Separation of Leaf Epicuticular Wax into Major Classes of Constituents by TLC.

Wax (1 mg) from each of the four cultivars of canola was spotted on a TLC plate, developed with benzene:hexane:chloroform (8:1:1), sprayed with 50% sulfuric acid and heated at 150°C for 15 minutes.
Nine bands were present for each cultivar, with about the same density for each cultivar.
When the TLC plates were only heated at 70°C, the bands were not charred as much, but showed up as colored bands under UV light, as indicated below.

A;Candle, B;Tobin, C;Altex, D;Westar

Band	Composition	Color under UV
1	alkanes	white to yellow
2	esters	yellow to orange
3	ketones	blue
4	aldehydes	yellow
5	sec-alcohols	yellow to orange
6	ketols	blue
7	prim-alcohols	yellow to orange
8	triterpenols	orange
9	fatty acids	yellow to brown

Figure 25

GLC-trace for Candle Leaf Epicuticular Wax

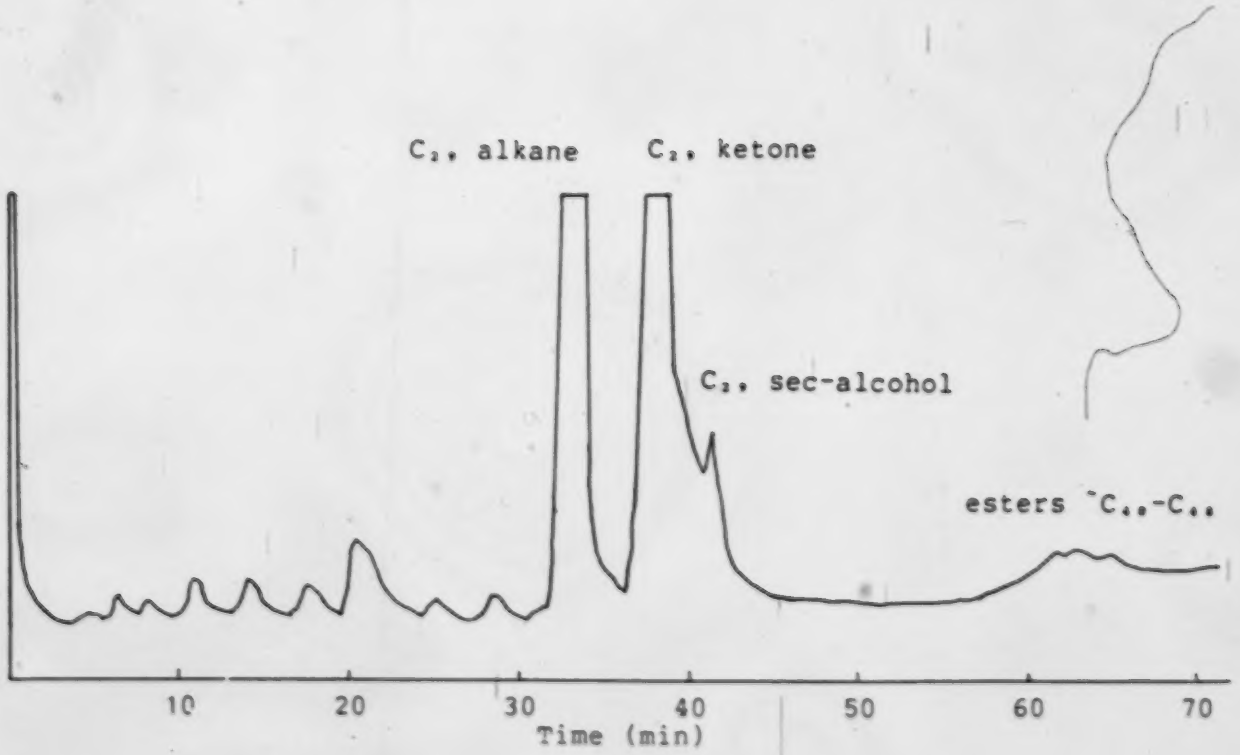


Figure 26

GLC-trace for Tobin Leaf Epicuticular Wax

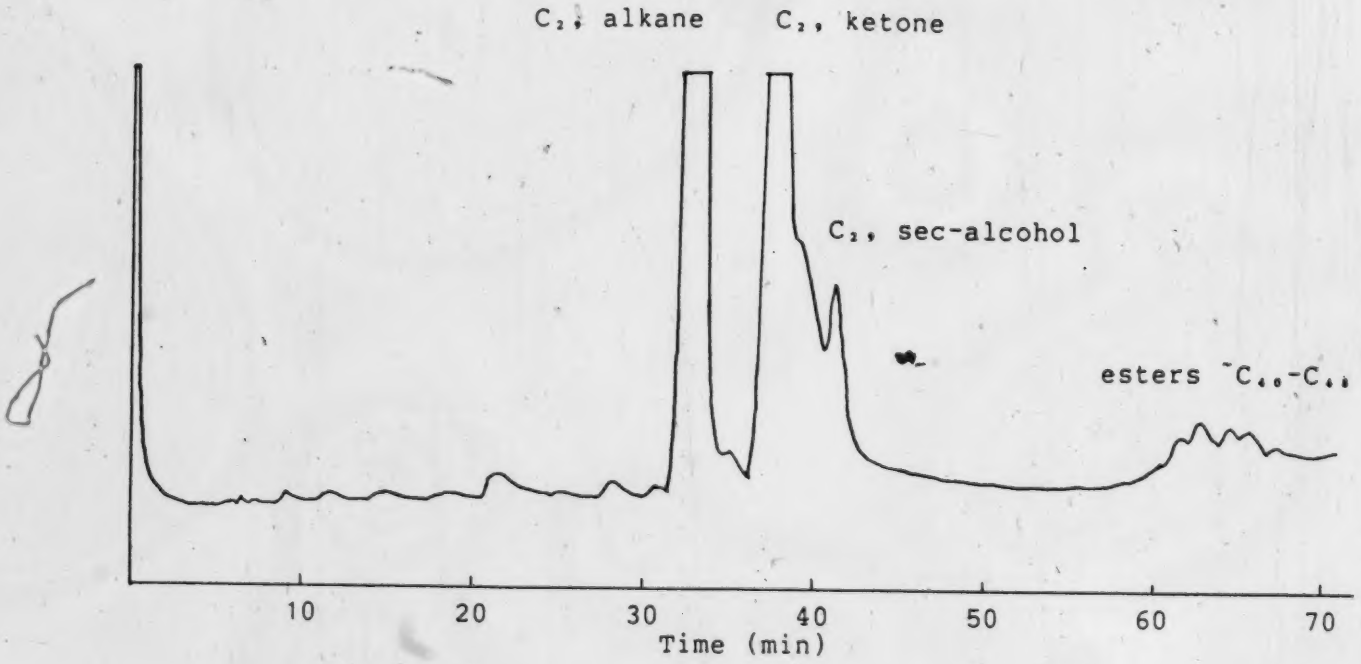


Figure 27

GLC-trace for Altex Leaf Epicuticular Wax

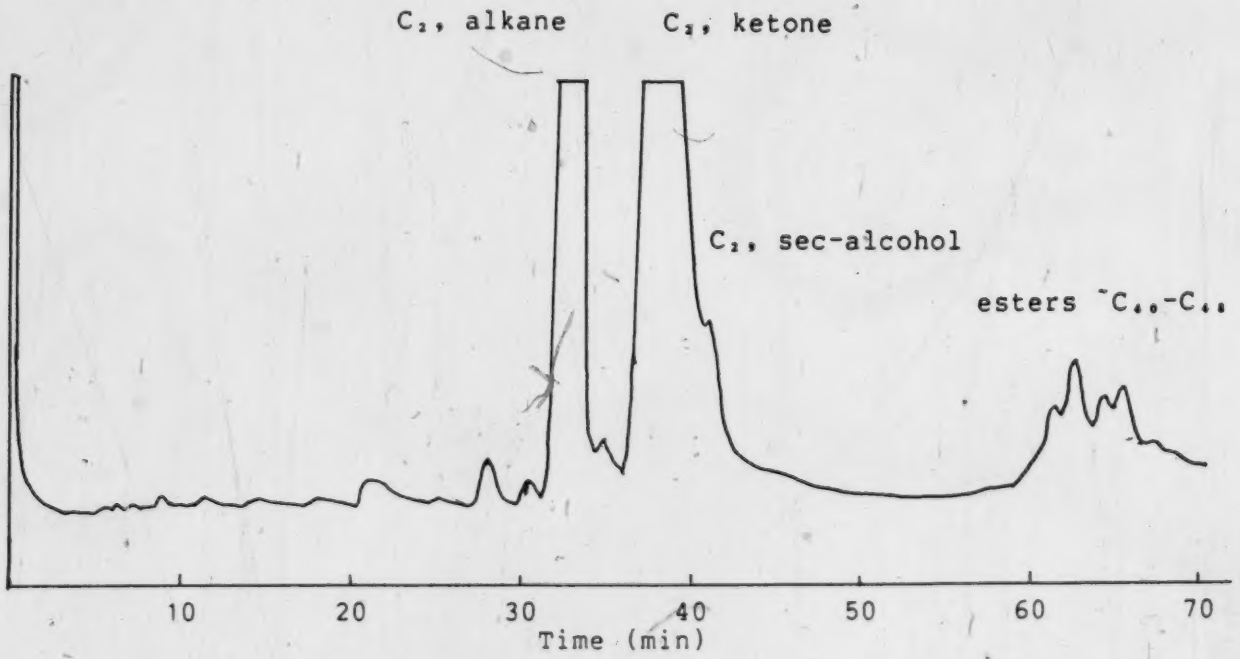


Figure 28

GLC-trace for Westar Leaf Epicuticular Wax

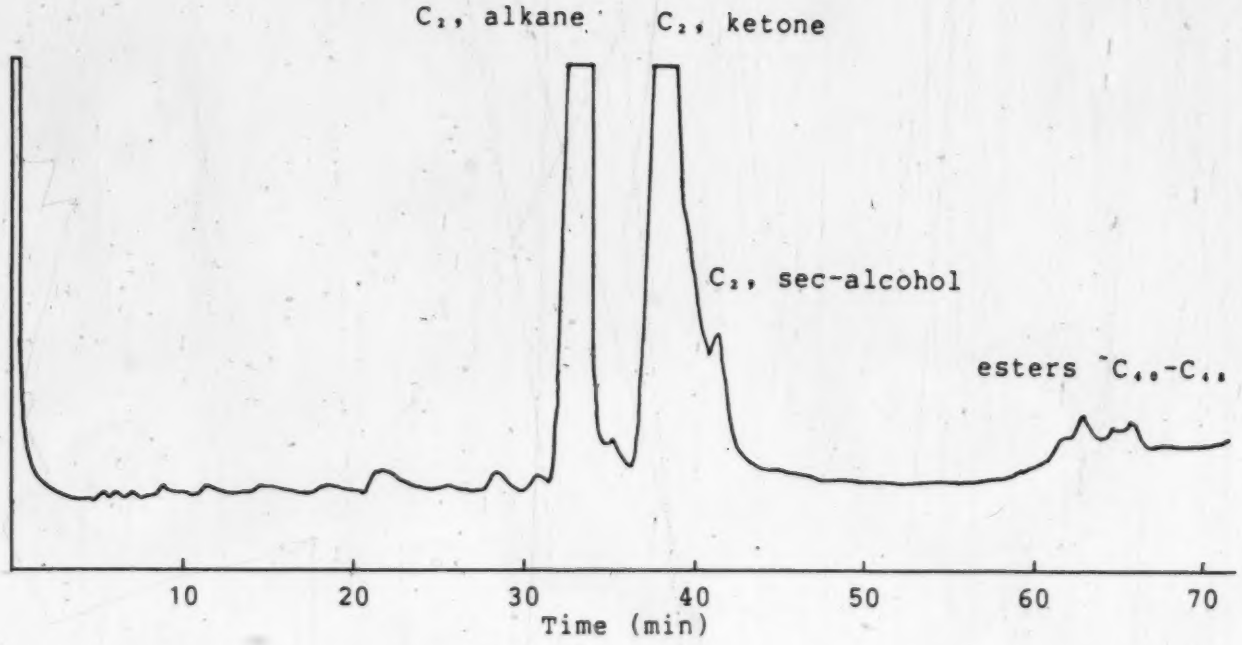


Figure 29

GLC-trace for the Alkane Fraction from Altex Leaf Epicuticular Wax

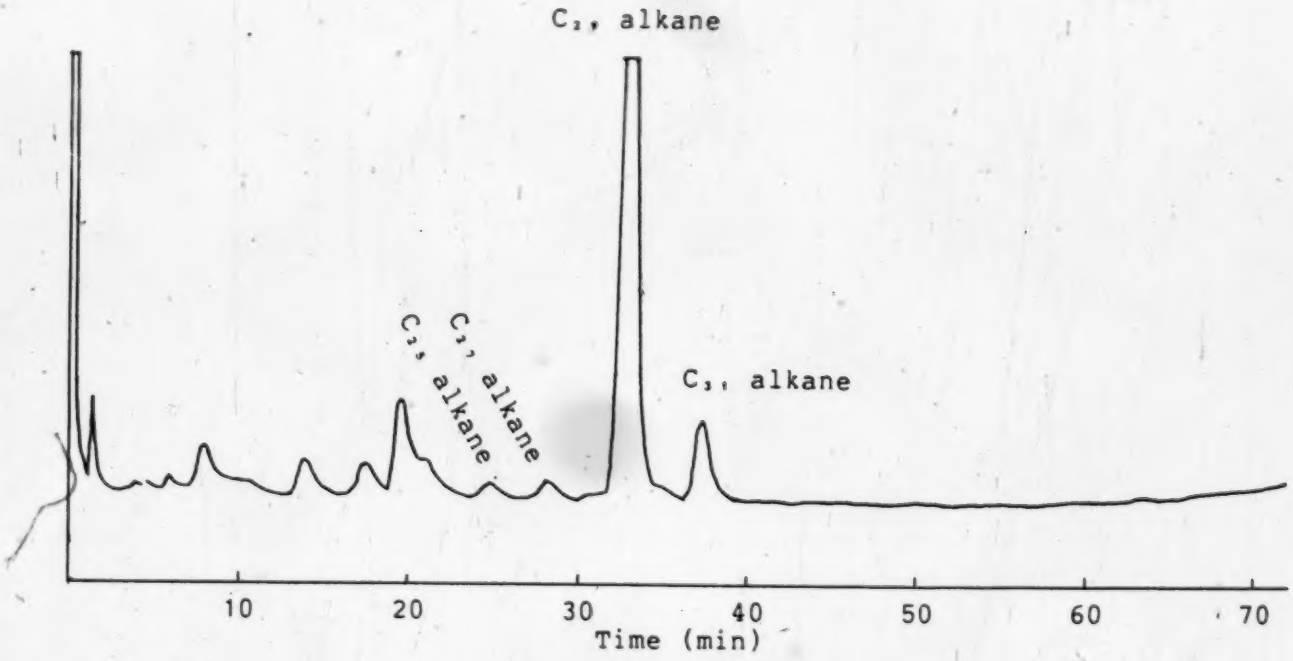


Figure 30

GLC-trace for the Ester Fraction from Altex Leaf Epicuticular Wax

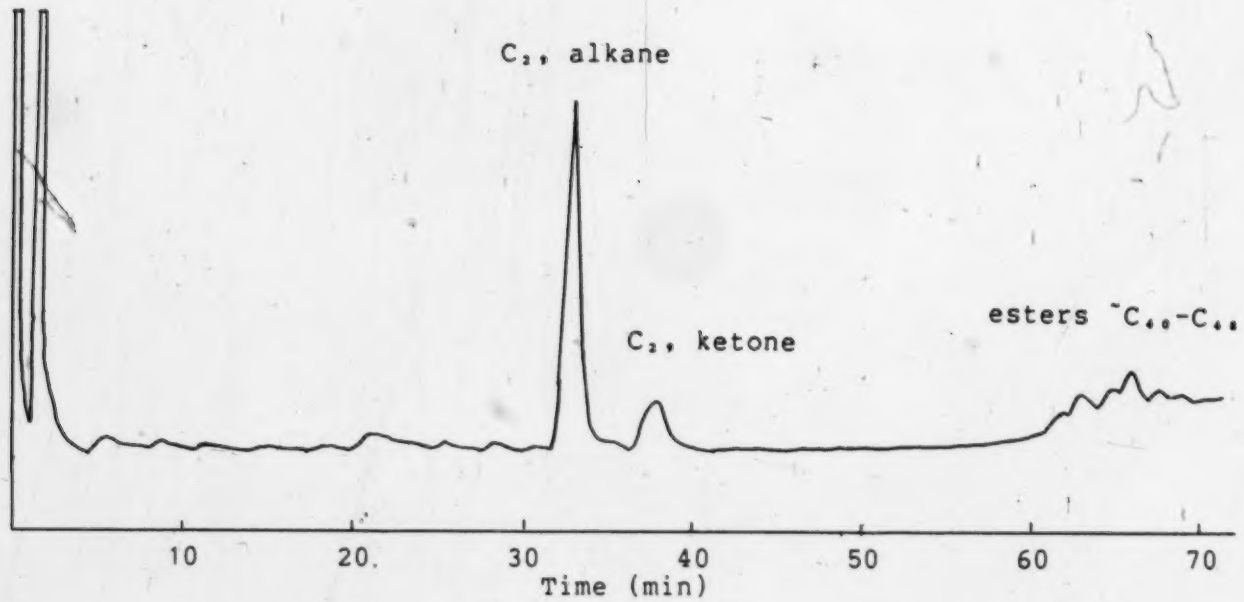


Figure 31

GLC-trace for the Ketone Fraction from Altex Leaf Epicuticular Wax

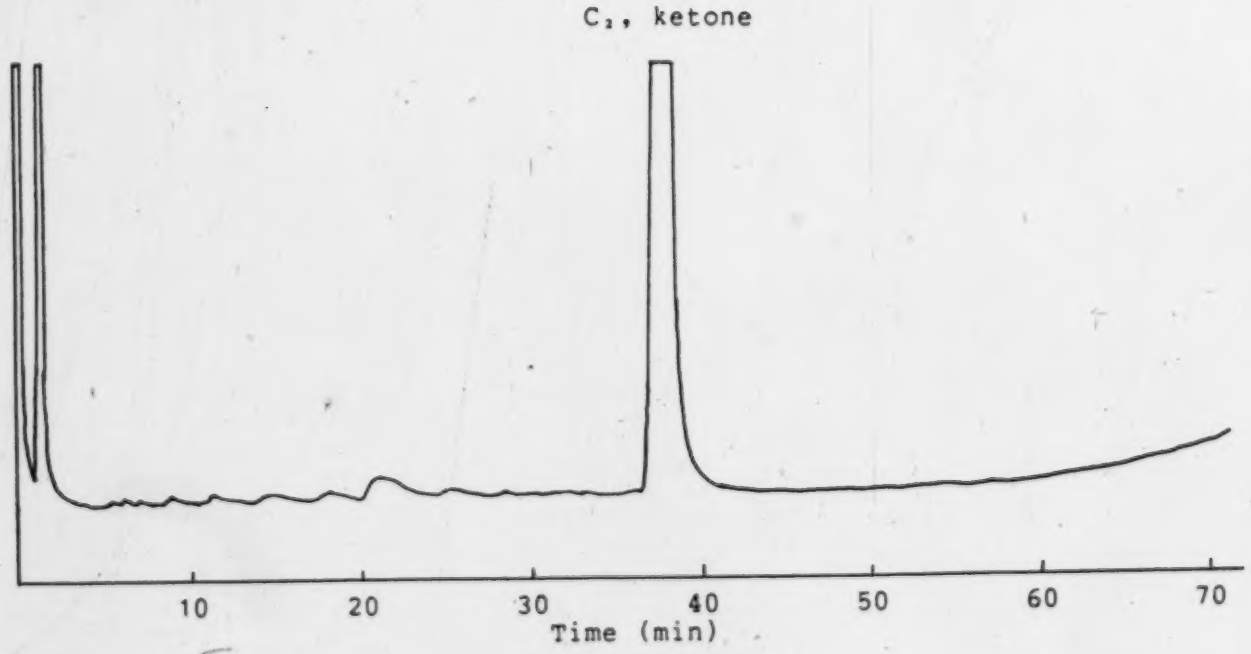


Figure 32

GLC-trace for the Sec-Alcohol Fraction from Altex Leaf Epicuticular Wax

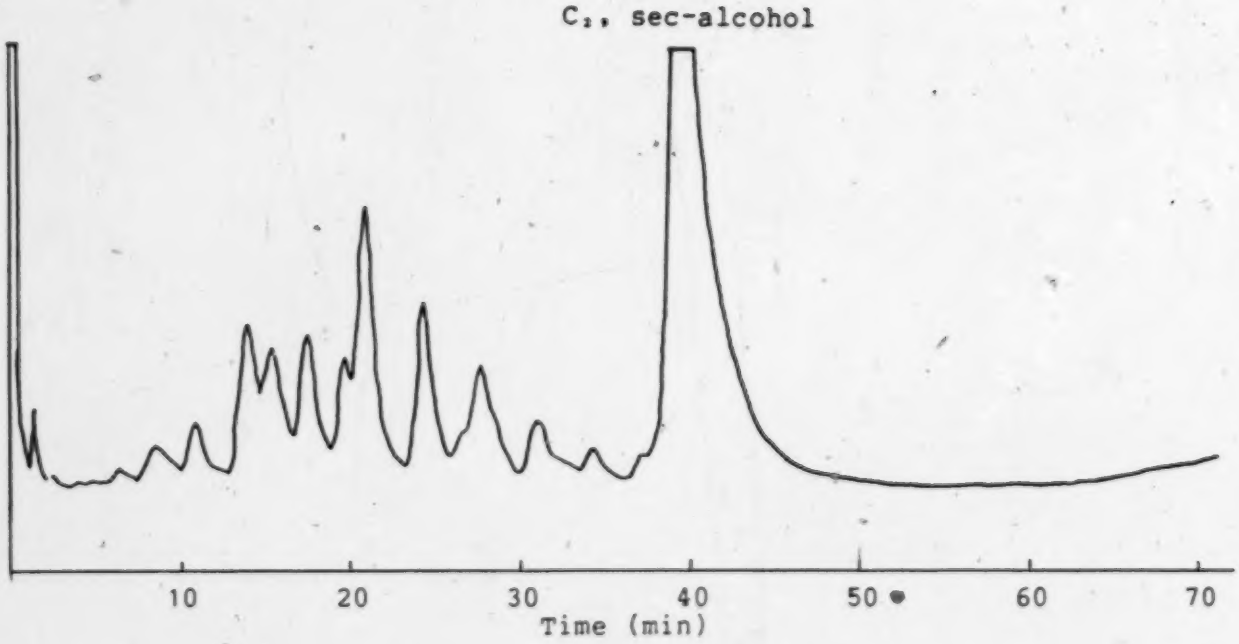


Figure 33

GLC-trace for the Aldehyde Fraction from Altex Leaf Epicuticular Wax

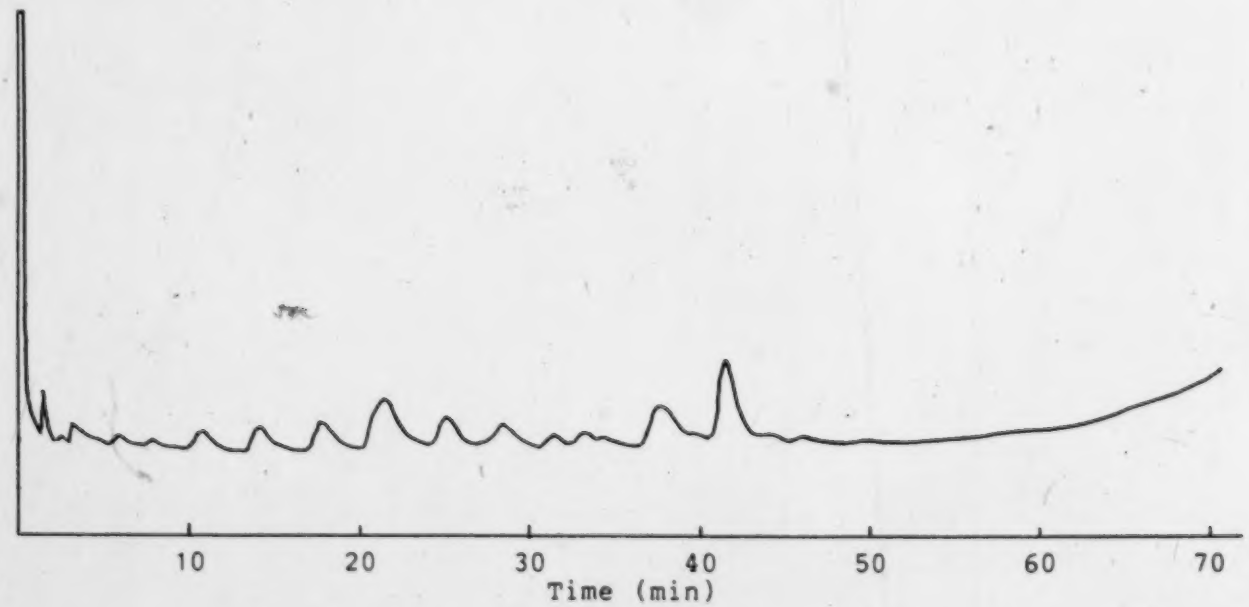
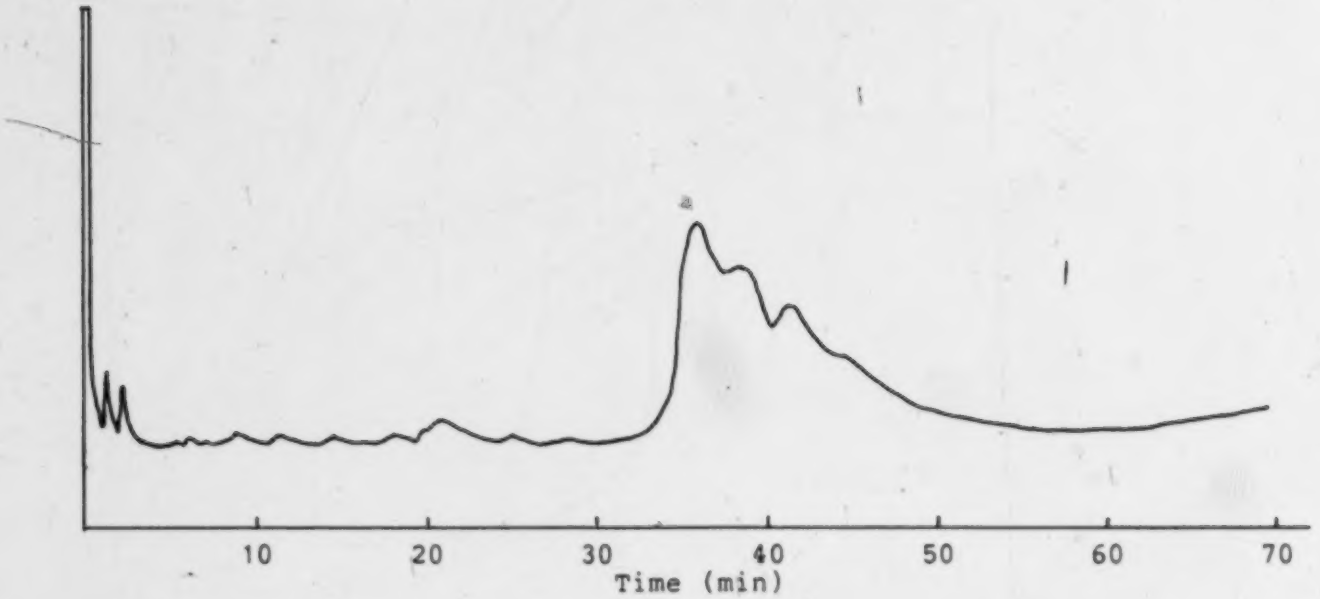


Figure 34

GLC-trace for the Prim-Alcohol Fraction from Altex Leaf Epicuticular Wax



Chapter V

General Discussion and Conclusions

A. Canola Epicuticular Wax versus A. brassicae

The goal of this project was to gather more information on the importance of epicuticular wax in conferring resistance in canola to A. brassicae. This information should be potentially useful in breeding cultivars of canola, especially species such as B. campestris that are susceptible to A. brassicae.

The leaf epicuticular wax of canola appears to confer lower susceptibility to A. brassicae in at least three ways. The first is that the wax creates a hydrophobic surface which decreases the retention of water-borne inoculum of A. brassicae. This factor, if considered alone, probably has the biggest impact on reducing susceptibility of the glaucous B. napus cultivars to A. brassicae. Two other phenomena must, however, be considered in this context. The first is that the surface of A. brassicae conidia are hydrophobic (Tewari, 1984), like that of the plant surface. The second is that Davis (1961) found that when a droplet carrying spores falls on and rolls over a hydrophobic plant surface, any hydrophobic spores floating on the surface of the droplet and brought into contact with the plant surface will tend to be deposited at the rear of the moving droplet, whereas hydrophilic spores (being submerged) will stay with the droplet. Since the conidia of A. brassicae are

hydrophobic one might expect more conidia to remain on the more hydrophobic surfaces. However, it was shown that this is not what happens, possibly because some droplets of water containing conidia bounce off the surface rather than landing and rolling off it. Also, most of the conidia of A. brassicae become submerged soon after they are suspended in water. Therefore, the hydrophobicity of most of the conidia is short-lived. Obviously, in this particular situation, the interaction of the water droplets and the hydrophobic surface outweighs the interaction of the initially hydrophobic conidia and the hydrophobic leaf surface.

There may also be an interaction between an hydrophobic leaf surface and dry air-borne spores. Forster (1977) studied the deposition of dry, air-borne spores of Lycopodium on the leaves of Sitka spruce. By comparing leaves with varying amounts of wax and leaves with the wax removed, Forster found that spore deposition was correlated with the amount of wax present. Forster proposed that this was due to the wax creating a rough surface, which reduced the bouncing off of dry air-borne spores.

The second way in which the leaf epicuticular wax of canola confers lower susceptibility to A. brassicae is that the germination rate of conidia is less when the leaves are glaucous. It was found that wiping the wax off the leaves increased the germinaton rate of conidia. This phenomenon is most likely due to the effect of wax on the diffusion of leaf exudates into droplets of water containing conidia.

Removal of the wax allowed a greater availability of leaf exudates, which may stimulate the conidia to germinate faster. The stimulatory effect of leaf exudates was also demonstrated by the fact that the germination rate of conidia on wiped glaucous leaves and on wiped or unwiped non-glaucous leaves was often higher than that in distilled water.

The third way in which the leaf epicuticular wax of canola confers lower susceptibility to A. brassicae is that the number of germ tubes produced by a conidium is less on glaucous leaves relative to that on the non-glaucous leaves. This phenomenon, like that of reduced germination rate, is most likely due to the effect of wax on diffusion of the leaf exudates. When the number of germ tubes is decreased, it should have an effect similar to having fewer conidia present. The fewer germ tubes a conidium produces, the less are the chances of infection occurring. With fewer germ tubes and slower germination of conidia on glaucous leaves, fewer conidia may have time to germinate and penetrate the leaf before the film or drop of water they are in dries up. This would likely be a very important factor in reducing infection by conidia present in the morning dew. The conidia would only have a few hours, at the most, to germinate and penetrate the leaf before the dew droplets and the conidia with them dry up.

Thus, when the effects of these three factors are added together the effect of epicuticular wax in conferring lower

susceptibility in B. napus to A. brassicae may be substantial.

It was also determined that the leaf epicuticular wax of canola has no fungistatic effect on the conidia of A. brassicae, even though the germination rate of conidia on glaucous leaves was sometimes less than that in distilled water. One possible explanation for this phenomenon could be that some conidia were deposited on the glaucous leaves without the presence of any appreciable quantity of water, and thus may have germinated slowly or did not germinate at all due to the rapid drying. This would decrease the percent germination in such a treatment and make it appear as if it was a fungistatic effect.

Thus, it has been demonstrated that the leaf epicuticular wax in canola is involved in conferring resistance to A. brassicae. A study of the ultrastructure, amount and chemistry of the wax was undertaken to determine if these aspects are important as well in regulating the susceptibility of canola to A. brassicae.

It was determined that there were no significant differences in the ultrastructure of wax in the four cultivars of canola studied. The surfaces of these canola cultivars were found to be covered with an evenly distributed layer of wax crystals superimposed on an amorphous layer of wax. There appeared to be at least three types of wax crystals present. These included plate-like crystals, filamentous, sometimes branched crystals, and

rods, present singly or forming blocks. The density of the wax crystals varied from cultivar to cultivar and from one part of the plant to another, but all the plant surfaces studied had the same types of wax crystals. An important aspect of the wax crystals was that many of them were projecting away from the plant surface, forming a "forest" of wax crystals. This is an important factor in the hydrophobic effect imparted by the wax. Also, when droplets of water sit on a leaf surface a pocket of air is trapped between the droplets and the leaf surface, which would decrease any exchange of exudates between the surface and the droplets.

Some trends such as the density of the wax on leaves and fruits appeared to be species specific, whereas the density of the wax on the stems did not. The density of the wax was high on the stems of all four cultivars. Also, the younger leaves of B. campestris often had a much higher density of wax than the older leaves. It was found that B. napus had on an average better than twice as much wax as that present in B. campestris.

The chemistry of waxes from the four cultivars was also studied. It was determined that the general chemical composition of the waxes is very similar for all four cultivars. The waxes consisted of nine major classes of constituents. These included alkanes, esters, ketones, aldehydes, sec-alcohols, ketols, prim-alcohols, triterpenols and fatty acids. The major constituents of the wax were C_{31},

alkane, C_{2}, ketone, C_{2}, sec-alcohol and ~C_{4}-C_{4} esters.

The only significant difference between B. napus and B. campestris was in the amount of epicuticular wax. This means that the effect of the wax on susceptibility to A. brassicae is due just to the amount of wax present and not to any differences in ultrastructure or chemistry. This indicates that in order to reduce susceptibility in canola to A. brassicae, plant breeders would only have to breed for increased wax production in B. campestris and not for any changes in ultrastructure or chemistry of the wax. Brassica campestris has the genetic potential to produce large amounts of wax, as evidenced by their glaucous stems, and their somewhat glaucous young leaves. The synthesis of leaf epicuticular wax in B. campestris appears to stop or slow down as the leaves age.

B. Suggestions for Future Studies

The effect of epicuticular waxes on plant processes such as photosynthesis and parameters such as growth and yield should be studied before a breeding program is initiated. Cameron (1970) studied the effects of various amounts of wax on photosynthesis using Eucalyptus leaves. He observed that increased wax deposits reduced the capability of the leaves to absorb light of wavelengths 400-700 nm. He noted that when the amount of light was below that required for light-saturated photosynthesis, leaves with the wax wiped off had higher rates of photosynthesis than did leaves with the wax intact. This indicates that glaucous plants may be at a disadvantage when grown in shaded areas. However, the amount of wax should not affect photosynthesis when there is plenty of sunlight.

A major area that needs to be examined is that of the leaf exudates and their effect on germination of conidia of A. brassicae. This thesis provided indirect evidence that the conidial germination is stimulated by leaf exudates. The role of leaf exudates may not be the same for all the cultivars of rapeseed. Sharma and Gupta (1978) studied leaf leachates from three cultivars of B. campestris grown in India. They found that the exudates inhibited the conidial germination of A. brassicae and A. brassicicola. Neither the amount of leaf exudates nor their composition are known for canola. A comparison needs to be made between B. napus and B. campestris to quantify and identify their leaf

exudates, since they appear to play a role in infection by A. brassicae.

Direct effects of wax on the movement of foliar exudates should also be studied. This could be done by monitoring the leaching of exudates on leaves with and without the wax wiped off. Also, model systems could be set up using isolated wax to study the diffusion impedance properties of the wax.

Another area that would be very interesting, is the recrystallization of wax and wax constituents to study the relationship between chemistry and ultrastructure of the wax. This area of research also has the potential of answering questions as to how the wax is transported to the plant surface.

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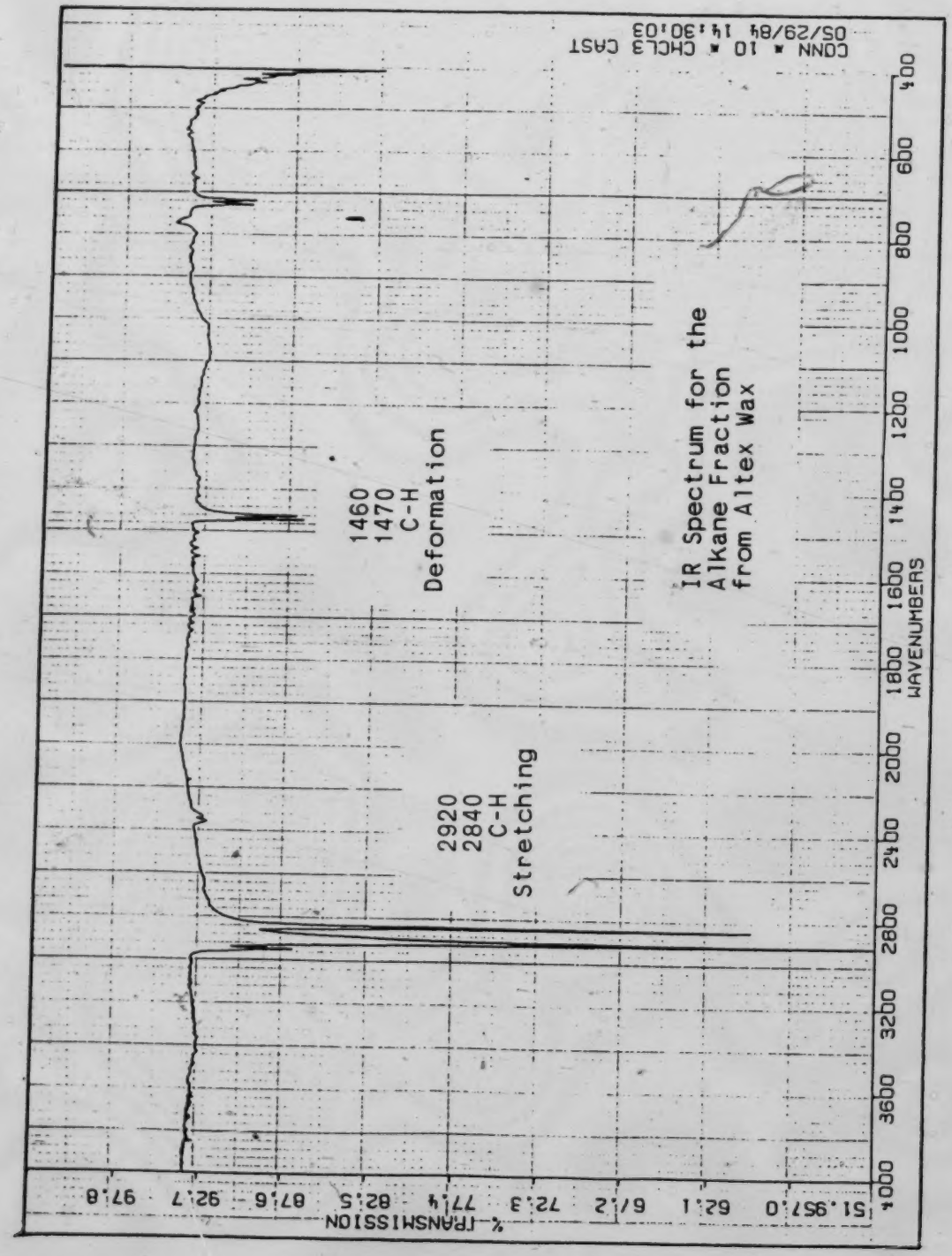
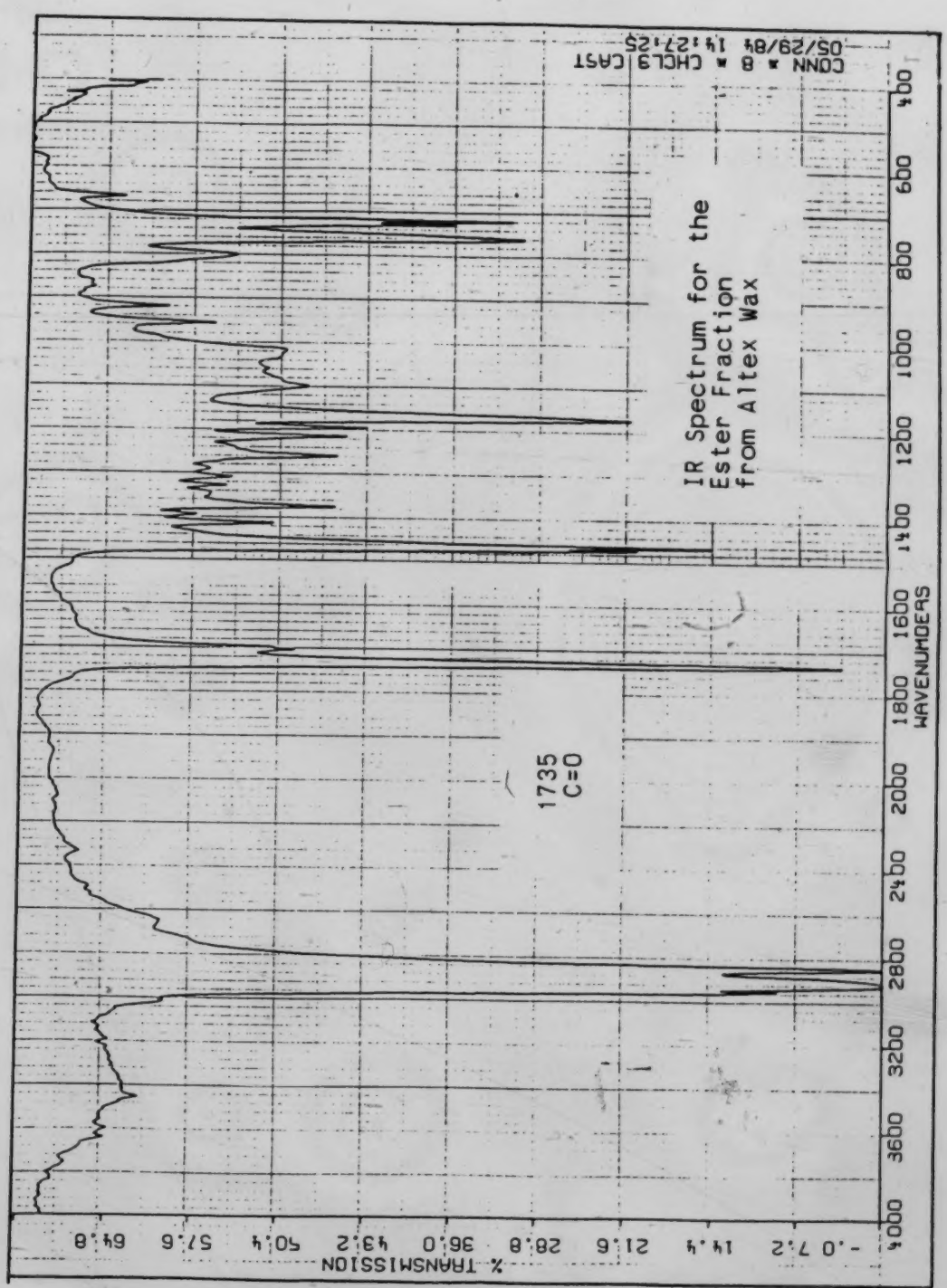
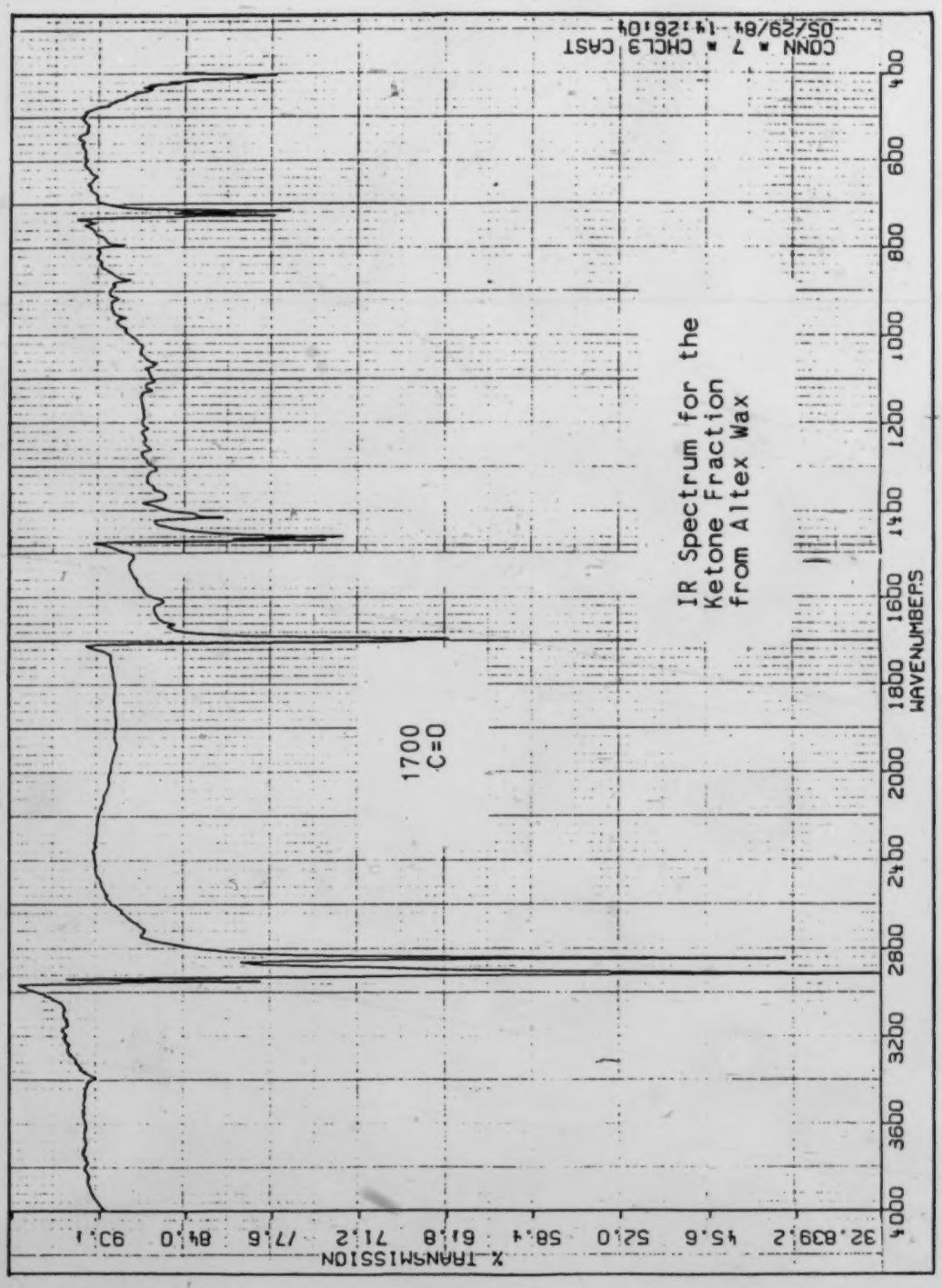
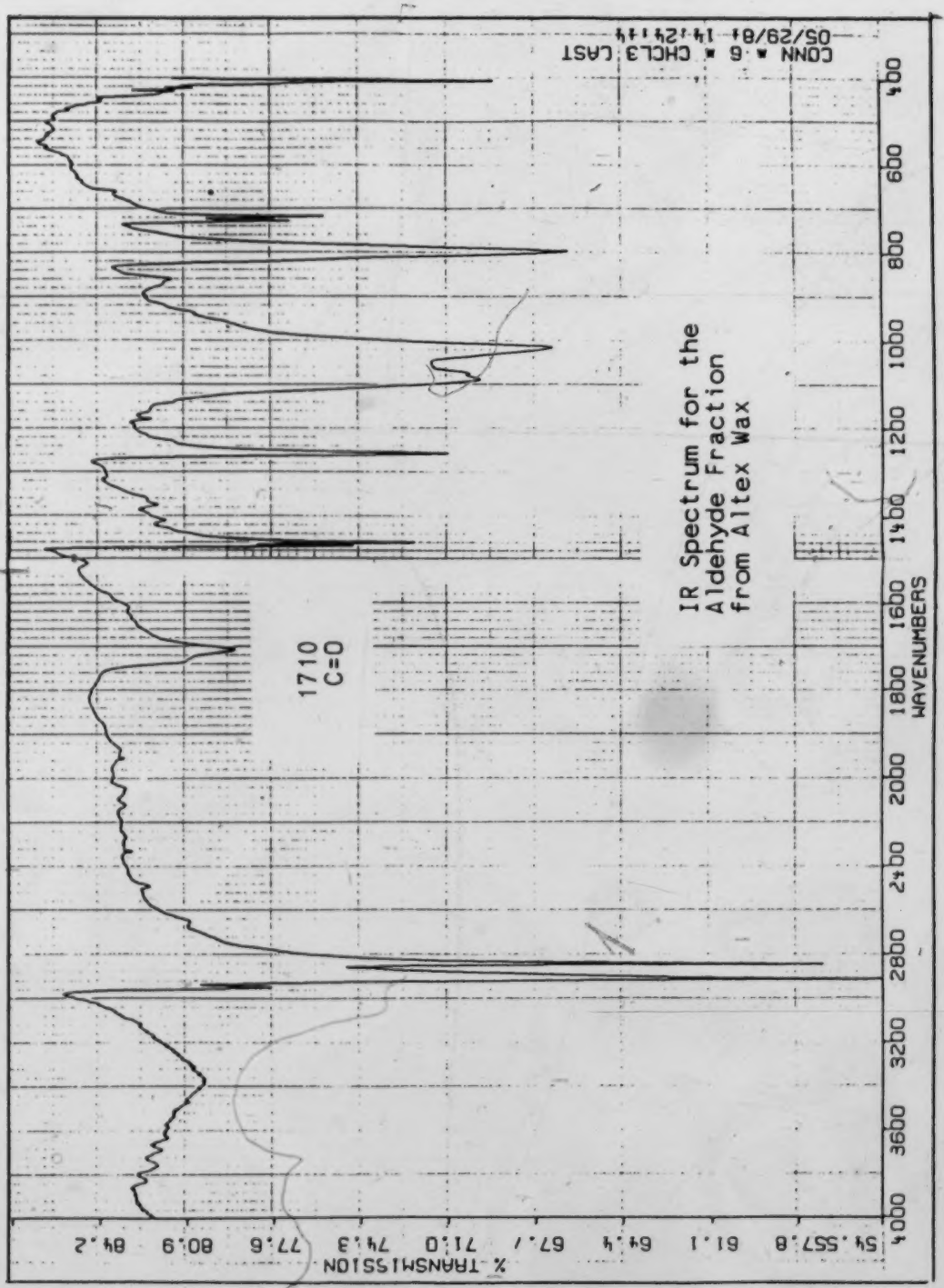
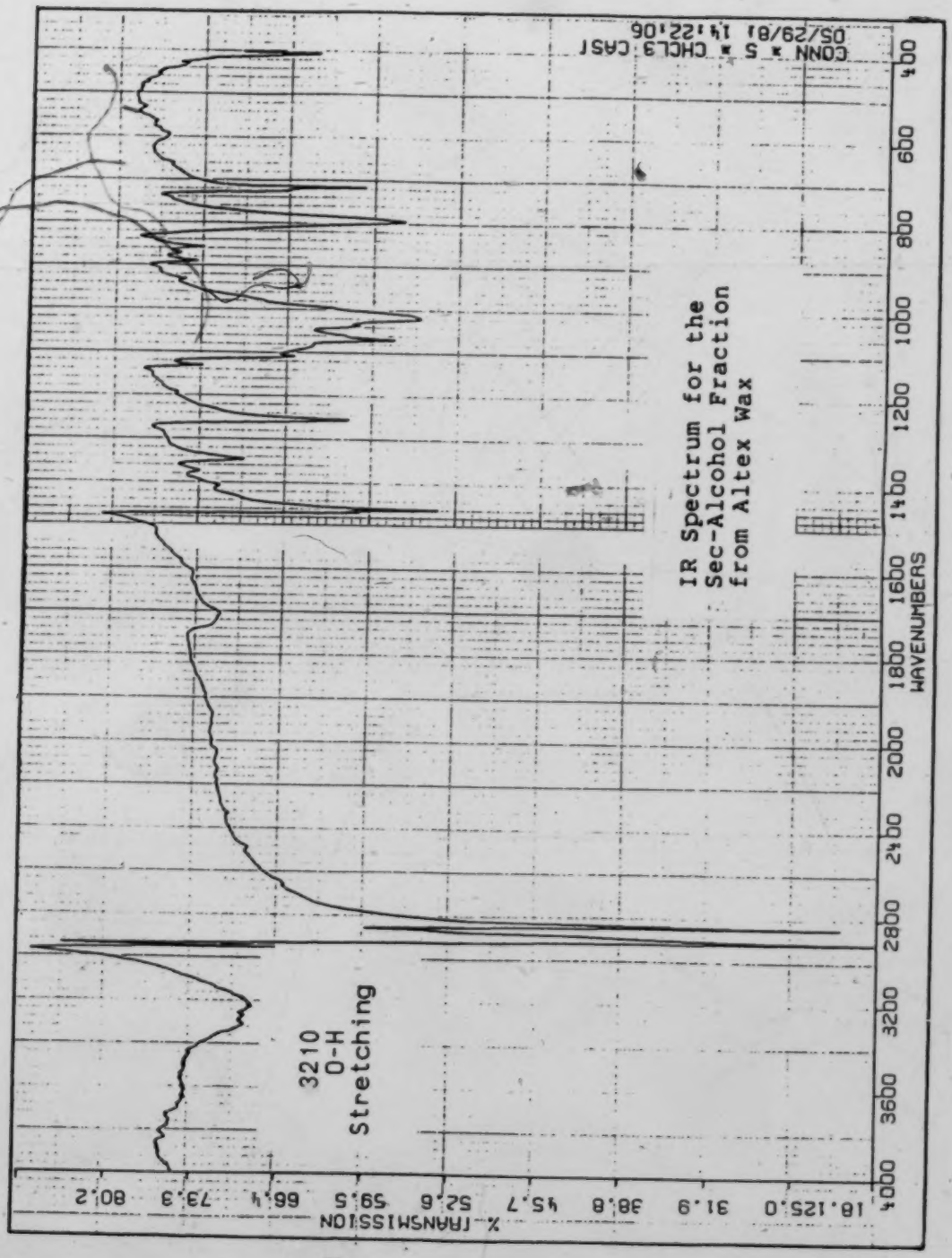
Appendix: Infrared Spectra for the Nine Classes of Constituents from the Fractionated Leaf Epicuticular Wax of Altex.

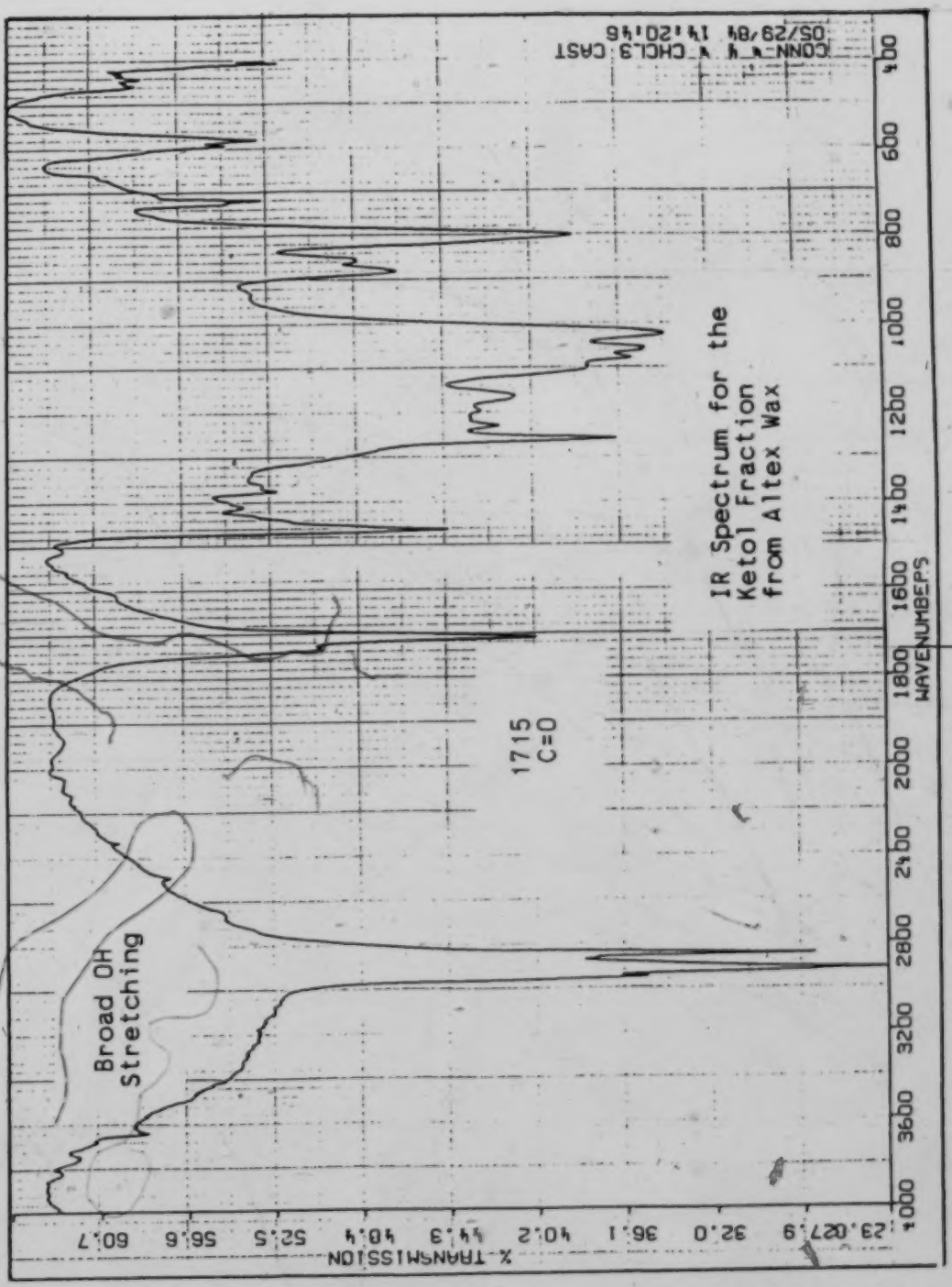

CONN = 10 = CHCL3 CAST
05/29/84 14:30:03
IR Spectrum for the
Alkane Fraction
from Altex Wax
1460
1470
C-H
Deformation
2920
2840
C-H
Stretching
WAVENUMBERS
% TRANSMISSION
51.9
57.0
62.1
67.2
72.3
77.4
82.5
87.6
92.7
97.8
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400

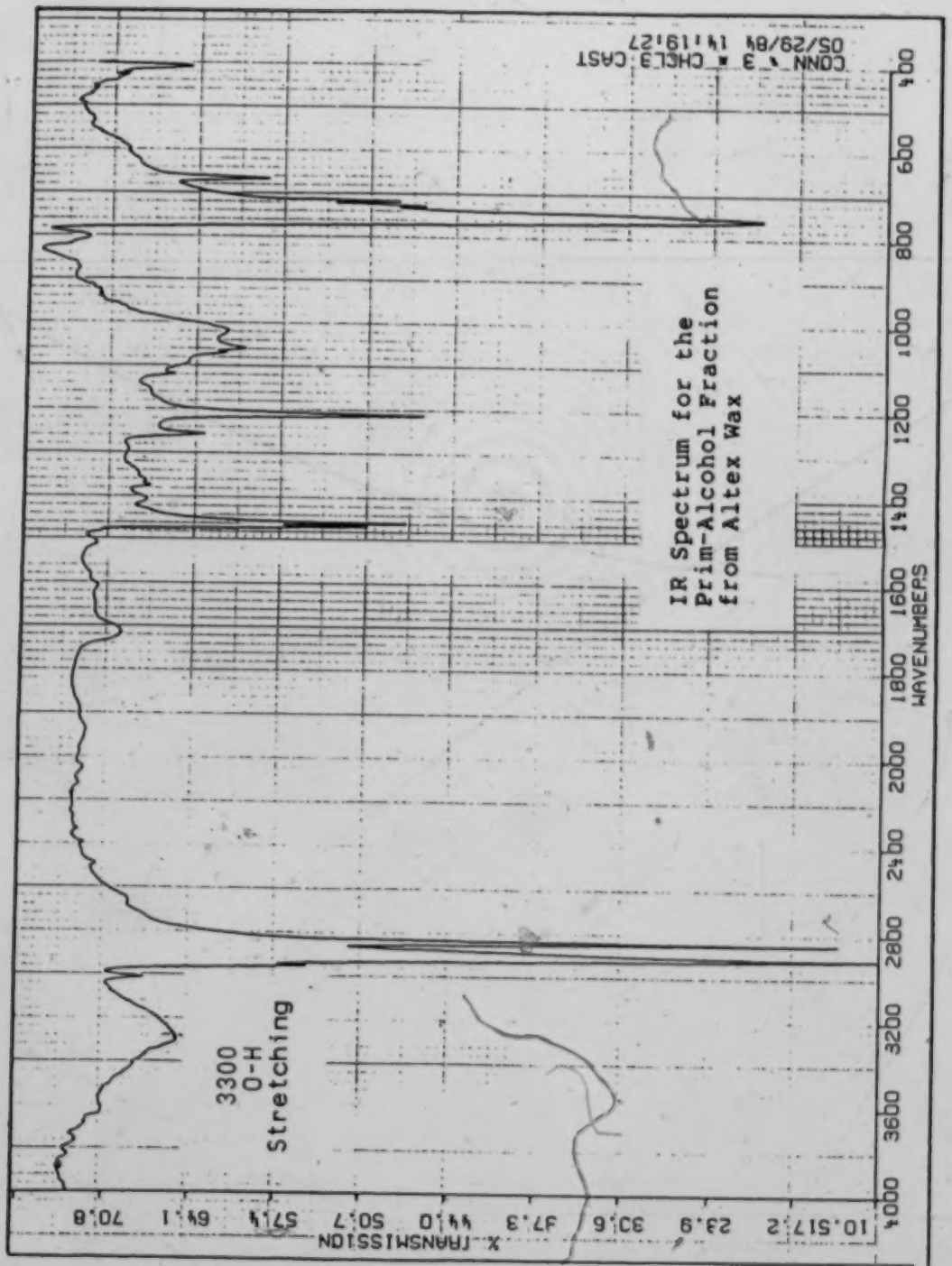

CONN * 8 * CHCL3 CAST
05/29/84 14:27:25
IR Spectrum for the
Ester Fraction
from Altex Wax
1735
C=O
% TRANSMISSION
-.0 7.2
14.4
21.6
28.8
36.0
43.2
50.4
57.6
64.8
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400
WAVENUMBERS

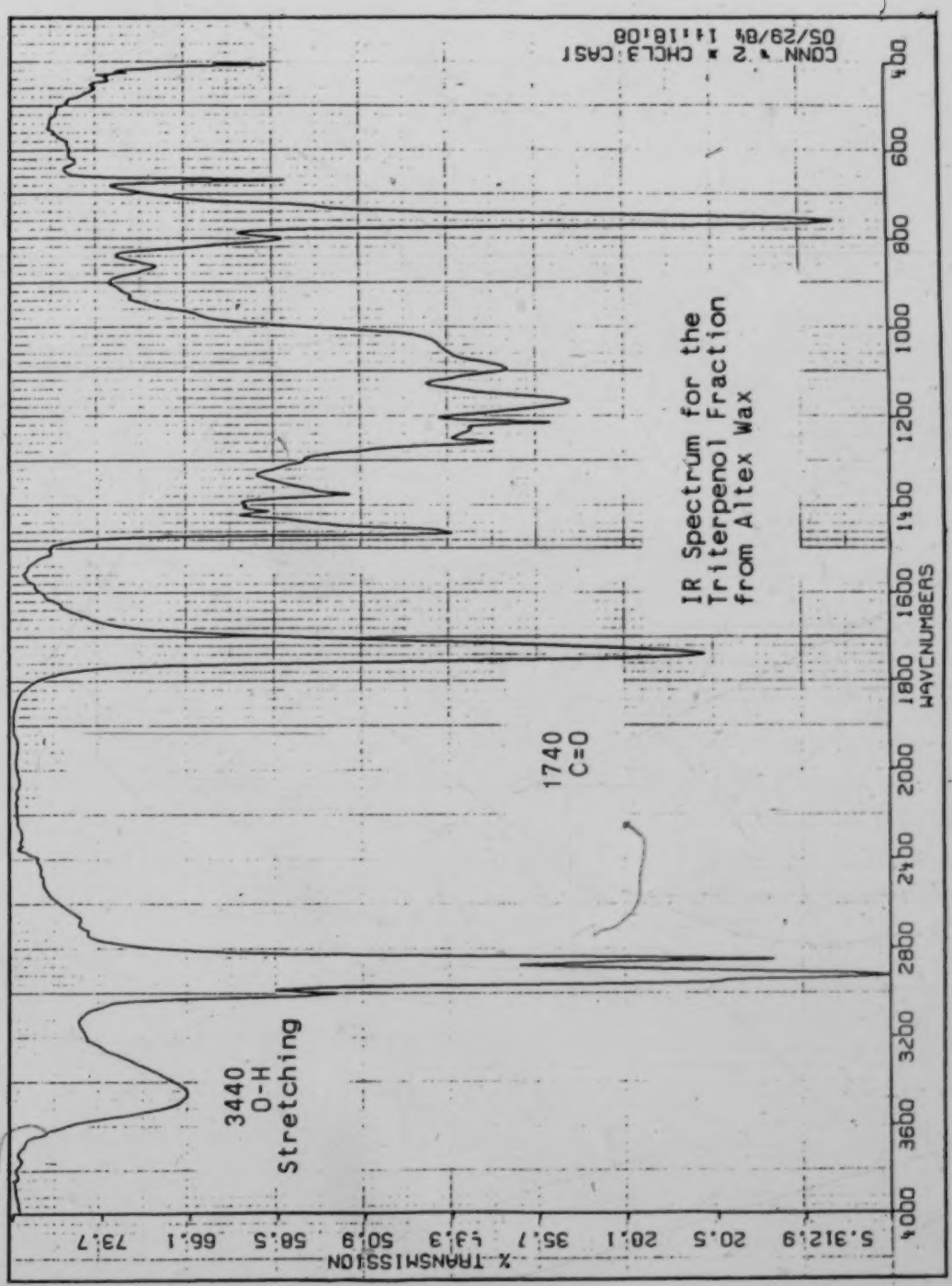

CONN = 7 = CHCL3 CAST
05/29/84 14:26:04
IR Spectrum for the
Ketone Fraction
from Altex Wax
1700
C=O
% TRANSMISSION
32.8
39.2
45.6
52.0
58.4
64.8
71.2
77.6
84.0
93.1
WAVENUMBERS
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400

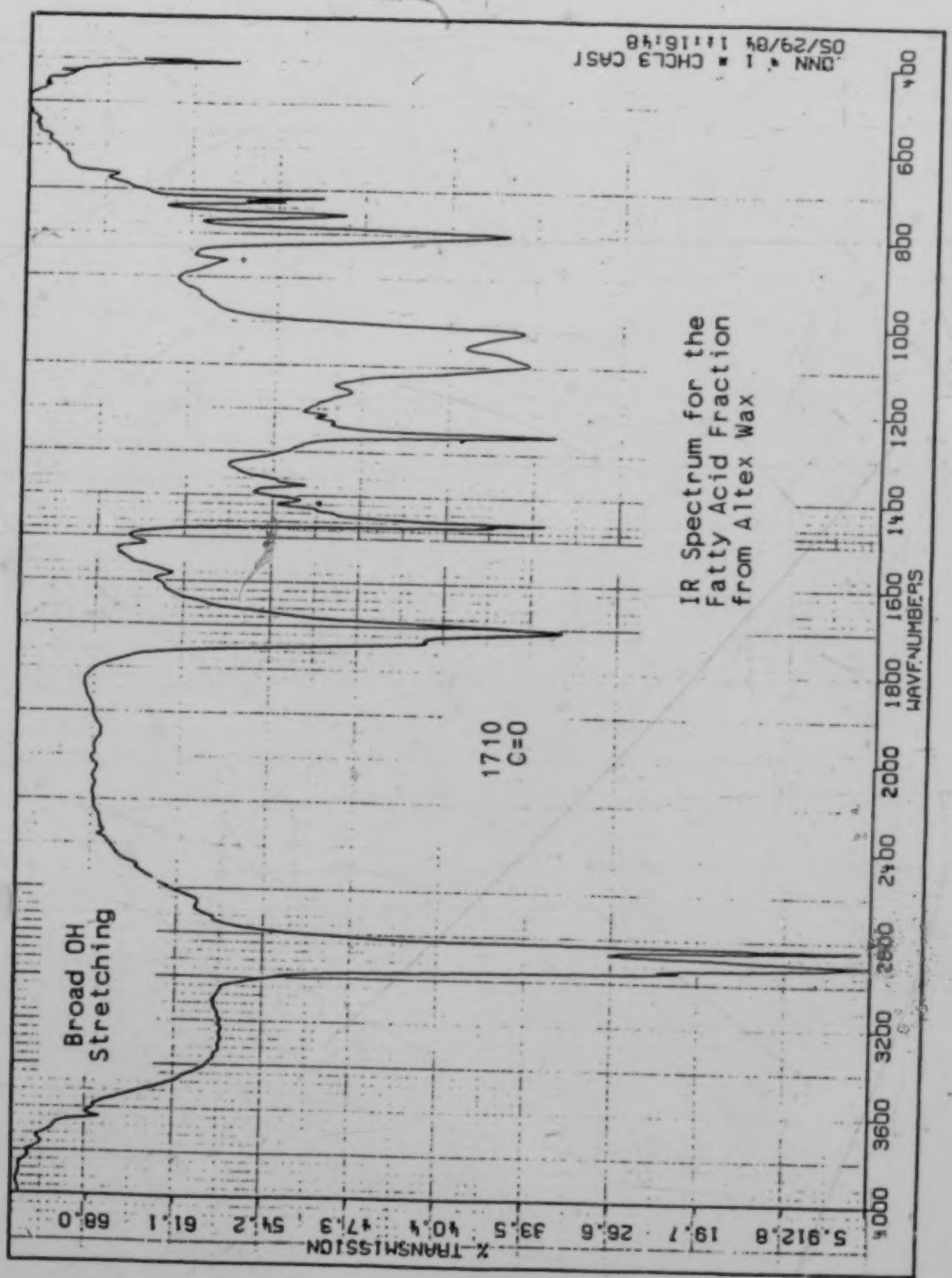

CONN ▲ 6 ▲ CHCL3 CAST
05/29/81 14:24:14
IR Spectrum for the
Aldehyde Fraction
from Altex Wax
1710
C=O
% TRANSMISSION
54.557.8 61.1 64.4 67.7 71.0 74.3 77.6 80.9 84.2
4000 3600 3200 2800 2400 2000 1800 1600 1400 1200 1000 800 600 400
WAVENUMBERS


CONN * S * CHCL3 CAST
05/29/81 14:22:06
IR Spectrum for the
Sec-Alcohol Fraction
from Altex Wax
3210
O-H
Stretching
% TRANSMISSION
18.125.0
31.9
38.8
45.7
52.6
59.5
66.4
73.3
80.2
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400
WAVENUMBERS


IR Spectrum for the
Ketol Fraction
from Altex Wax
Broad OH
Stretching
1715
C=O
WAVENUMBERS
% TRANSMISSION


IR Spectrum for the
Prim-Alcohol Fraction
from Altex Wax
3300
O-H
Stretching
% TRANSMISSION
10.5 17.2 23.9 33.6 37.3 44.0 50.7 57.4 64.1 70.8
4000 3600 3200 2800 2400 2000 1800 1600 1400 1200 1000 800 600 400
WAVENUMBERS
CONN \ 3 ▪ CHCL3 CAST
05/29/84 14:19:27


CONN = 2 * CHCL3:CAST
05/29/84 14:18:08
IR Spectrum for the
Triterpenol Fraction
from Altex Wax
3440
O-H
Stretching
1740
C=O
% TRANSMISSION
5.312.9
20.5
20.1
35.7
53.3
50.9
58.5
66.1
73.7
WAVENUMBERS
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400


IR Spectrum for the
Fatty Acid Fraction
from Altex Wax
Broad OH
Stretching
1710
C=0
WAVENUMBERS
% TRANSMISSION